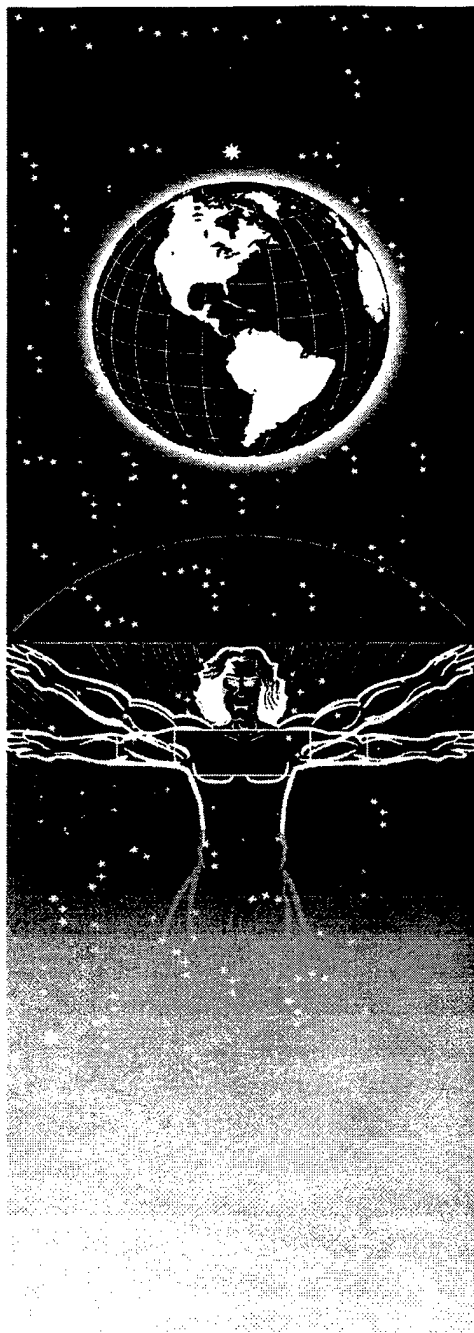AL/EQ-TR-1995-0030

UNITED STATES AIR FORCE
ARMSTRONG LABORATORY

Nonrecombinant Genetic Modification of Aquifer Bacteria to Achieve Constitutive Degradation of Trichloroethylene

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Aquifer bacteria were isolated for their capacity to predominate following nutrient enrichment (field application vectors (FAVs)), and screened as hosts for the Tn5 containing, constitutive toluene *ortho*-monoxygenase (Tom) expressing plasmid: TOM_{31c} (which causes the cooxidation of trichloroethylene (TCE)). Tom expression during positive selection in native aquifer sediments contaminated with TCE was determined. Three such FAVs were constructed: NFG-2 (TOM_{31c}), MFI-1 (TOM_{31c}), and MFG-2 (TOM_{31c}). All stably maintained TOM_{31c} and constitutively degraded TCE. Like the original TOM_{31c} mutant strain (*Burkholderia cepacia* G4 $PR1_{31}$), MFG-2 (TOM_{31c}) was unable to significantly degrade TCE in native sediments despite inoculation to high levels ($\geq 1 \times 10^8$ cells/gram), and did not apparently survive well in glucose amended material. NFG-2 (TOM_{31c}) and MFI-1 (TOM_{31c}) did significantly degrade TCE in glucose and IGEPAL (respectively) amended aquifer sediments. Like $PR1_{31}$ (TOM_{31c}), Tn5 gene probes indicated that these FAVs also remained above 10^6 cells per cm^3 of sediment during a 20 day feeding and TCE degradation experiment. TOM_{31c} was transferable to these selectable FAVs by non-recombinant mating techniques, and once there encoded constitutive TCE degradation in native sediments untreated save for the addition of a specific carbon and energy sources.

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PREFACE

This report covers research conducted during the period June 1994 through August 1995 by the University of West Florida Center for Environmental Diagnostics and Bioremediation, Department of Biology. The work was conducted under contract DAAL03-91-C-0034 issued by the Army Research Office and was funded by the US Air Force Armstrong Laboratory, Environics Directorate, Tyndall AFB Florida;. The Project Officer was Alison Thomas, (904) 283-6303.

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EXECUTIVE SUMMARY

A.   OBJECTIVE

The purpose of this document is to provide personnel involved in environmental restoration information about bacterial strains created through this research that are now available for the constitutive bioremediation of trichloroethylene (TCE). It is also intended to impart an understanding of the reasoning surrounding the development and selection criteria for such strains as well as data to assess their applicability to specific TCE-contaminated sites.

B.   BACKGROUND

Recent attempts to utilize bacteria ($PR1_{23}$ and $PR1_{31}$) capable of the degradation of TCE without inducer substrate (i.e. though the constitutive expression of the aromatic oxygenase: toluene *ortho*-monooxygenase (Tom)) indicated the capacity for cometabolic degradation of TCE under environmental conditions, but not necessarily stable expression by these strains. The location of these genes on the self-transmissable plasmid TOM creates certain possibilities for the non-recombinant transfer of these genes to other aquifer bacteria more capable of environmental function than our strain of *Burkholderia cepacia*.

C.   SCOPE

Recent literature has indicated the possibility of selecting bacteria capable of a selective growth response in such native materials when fed selective carbon sources. These field application vectors (FAVs) have been proposed as functional bioremediative vehicles for genetic elements containing cloned genes, and there is every reason to suppose that such vectors could also be isolated that would retain TOM (the catabolic plasmid encoding Tom) and also constitutively degrade TCE in the environment. Section II describes the isolation and screening of such strains from TCE-contaminated and uncontaminated aquifer material. Section III describes the transfer of a transposon marked version of TOM to several of these strains and the resultant capacity to degrade TCE. Section IV describes our survey of the TCE constitutive FAVs thus created to degrade TCE in the laboratory in native aquifer material experimentally contaminated with TCE.

D.   METHODOLOGY

Bacteria from several aquifers were isolated for their demonstrated capacity to either naturally predominate in the native sediments or predominate following a nutrient enrichment and

examined for their potential to serve as hosts for the Tn5 containing plasmid TOM_{31c} (which specifies the constitutive Tom production and cooxidation of TCE, dichloroethenes and vinyl chloride). These hosts were examined for their ability to express Tom and undergo positive selection in native aquifer sediment materials when experimentally contaminated with TCE and fed selective carbon sources.

E.   TEST DESCRIPTION

Transgenic bacteria created in this non-recombinant fashion (i.e. standard bacterial matings) were examined for their ability to express Tom constitutively by two methods. One was the capacity to oxidize a fluorinated analog of *ortho*-cresol, and the other was the ability to degrade TCE without aromatic induction as determined by air headspace or direct extraction analysis by gas chromatography.

F.   RESULTS

Three field application vectors (FAVs) were constructed in this manner: NFG-2 (TOM_{31c}), MFI-1 (TOM_{31c}), and MFG-2 (TOM_{31c}) were found to stably retain TOM_{31c} and constitutively degrade TCE under defined conditions. Tests in native materials indicated that like the parent strain (*Burkholderia cepacia* G4 $PR1_{31}$ (TOM_{31c})) MFG-2 (TOM_{31c}) was unable to significantly degrade TCE in native sediments. However, unlike $PR1_{31}$ (TOM_{31c}), MFG-2 (TOM_{31c}) did not apparently survive well in carbon amended materials (lactate and glucose respectively). The other two constructed strains: NFG-2 (TOM_{31c}) and MFI-1 (TOM_{31c}) did significantly degrade TCE in carbon amended aquifer sediment (glucose and IGEPAL respectively). Like the $PR1_{31}$ (TOM_{31c}), these effective degraders were also shown to maintain themselves (via Tn5 gene probes) at populations above 10^6 cells per cm^3 of sediment slurry during a 20 day feeding and TCE degradation experiment.

G.   CONCLUSIONS

TOM_{31c} was transferred to selectable FAV strains by non-recombinant mating techniques and, once there, found to be capable of the constitutive degradation of TCE in native sediment untreated save for the application of selective carbon and energy sources not inducing for TCE cometabolism.

H.   RECOMMENDATIONS

It is now clearly feasible to construct FAVs either tailored to a specific site and selective nutrient combination or to generate a pool of such organisms that can be used to obtain the "best" candidate for a given site. The strains described here should only be viewed as "proof of concept", and not necessarily the "best" attainable.

Other more selectable candidates should be sought and their suitability as stable hosts for TOM_{31c} determined. Also, the selectable hosts identified so far (NFG-2 and MFI-1) should next be transformed with the non-Tn5 containing constitutive TCE degrading plasmid TOM_{301c}, which is without transposon insertion and therefore more amenable to field release. This should in turn be tested further for the most ideal selective conditions.

This study has merely explored the most obvious conditions and modes of delivery. A more rigorous aquifer simulation needs to be used to evaluate the true potential of these or other transconjugants.

One of the more puzzling features of $PR1_{31c}$ is the apparent contradiction between its apparently good survivability and its relatively poor activity *in situ*. The two transconjugants that survived well (as determined by Tn5 hybridizable colonies) consequently were capable of extended TCE degradation as well. The poor survivor also (not surprisingly) did not degrade TCE. These results point out the need to better understand the physiology of the bacterium expected to perform the cometabolic task under the conditions it will have to perform it. Clearly survival is not an adequate predictor of performance.

Given the alternatives for remediation of TCE-contaminated aquifers such survivable, selectable, constitutive degraders should prove of great value if bioaugmentation is considered. Information gathered on the physiological state of such bacteria *in situ* would be of use in any bioaugmentation scenario.

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INTRODUCTION

A.   OBJECTIVES

The goal of this investigation was to isolate bacteria with a greater potential for environmental survival and selection than *Burkholderia cepacia* $PR1_{31}$, transfer the constitutive toluene *ortho*-monooxygenase (Tom) producing plasmid (TOM_{31c}) (9) to these alternate hosts, and screen them for phenotypic expression. These field application vectors (FAVs) were then to be tested in the laboratory to assess whether they would be any more stable or selectable in aquifer sediments than the parent organism: $PR1_{31}$. A further goal of this investigation was to provide strains proficient for field application and maintenance of TOM_{31c} which could then serve as hosts for TOM_{301c} (another Tom constitutive version of TOM that was created through nitrosoguanidine mutagenesis, and therefore not subject to EPA regulation as TOM_{31c} is due to the presence of Tn5). Such isolates containing TOM_{301c}, instead of TOM_{31c}, would then also be free from EPA regulation (being totally free of anything that could be construed as "recombinant") and therefore available for *in situ* testing.

B.   BACKGROUND

1.   Statement of the Problem

Recent attempts to utilize TOM constitutive strains $PR1_{23}$ and $PR1_{31}$ for the degradation of TCE in bioreactors and soil columns indicate a basic problem with the maintenance of these strains in the presence of native biota in waters and sediments. Recent literature has indicated the possibility of selecting bacteria capable of a selective growth response in such native materials when fed selective carbon sources. These FAVs have been proposed as functional bioremediative vehicles for genetic elements containing cloned genes (5,6). There is every reason to suppose that such vectors would retain TOM and in the case of the constitutive derivatives: TOM_{31c} and TOM_{23c} also express the TCE cometabolic Tom without inducer. Further difficulties with the release of these strains is the continued insistence by EPA that bacteria containing either of these two TOM derivatives be considered "significantly altered organisms" due to the presence of Tn5, and therefore subject to the same regulations as "recombinant organisms." Recent developments in our laboratory have resulted in the

production of a similar constitutive plasmid: TOM_{301c}, that was derived by purely chemical mutagenic means (publication in preparation).

2.   Envisioned Role of a Constitutive TCE Degrader in Aquifer Remediation and Need for this Technology.

Current technologies for the treatment of TCE-contaminated soil and water have relied primarily upon pump-and-treat systems, whereby TCE is distilled away from the water under vacuum, or alternatively, is air-stripped and transferred, or sorbed directly, onto an adsorbent such as charcoal. In either event, the end result is the production of more polluted material or atmosphere. The capability to destro·· the contaminant at the site would represent significant environmental and economic benefits. Bioremediation technologies would fill this need.

3.   Biochemistry

Toluene catabolism by G4 has been shown to proceed via a monooxygenation pathway that results first in an *ortho*-hydroxylation of toluene and then in *ortho*-hydroxylation of the resultant cresol to form 3-methylcatechol (11).
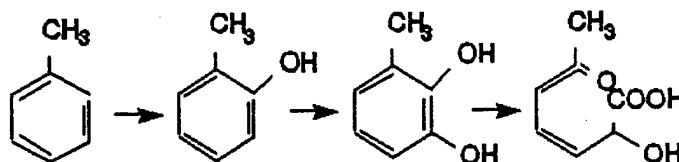
This pathway is utilized for growth on toluene, phenol, benzene, cresol and xylene isomers by G4. Studies of various mutants of this pathway have revealed that the function required for the hydroxylation of toluene and phenol is also that required for the oxidation of TCE (12). The limitations of these bacterial systems for the bioremediation of TCE in the environment is that all natural isolates degrade TCE fortuitously. Their ability to alter TCE is necessarily linked to the production of an enzyme that can coincidentally accept TCE as an alternative substrate, the native substrate being that which is used to induce its synthesis. Due to this co-metabolic relationship TCE cannot be degraded in the environment without the addition of an exogenous inducing substrate. As a result these organisms are faced with the task of degrading TCE only when in the presence of an inducing substrate that must compete for the

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same active site on the induced enzyme. This also means that the organisms are not active beyond the environmental zone that can be controlled through the addition of effective concentrations of inducer: i.e., the bacteria are essentially "tethered" to the inducing substrate. Both of these limitations have serious implications in both environmental and bioreactor applications. In addition the application of the native inducing substrates such as toluene or phenol to an already contaminated environment is difficult because they are regulated pollutants.

The capacity of $PR1_{23}$ and $PR1_{31}$ (two Tom constitutive derivatives of G4 described below) to degrade TCE under environmental conditions was recently demonstrated for bioreactors and soil columns. This study indicated the inherent capacity of this bacteria and its enzyme system to degrade TCE under environmental conditions; it also demonstrated the inherent instability of these strains, and pointed to the obvious advantage of first selecting a more appropriate host for an apparently environmentally functional biochemical mechanism.

4.   Development of a Constitutive TCE Degrader

Transposon-induced mutants of *B. cepacia* G4, unable to grow with phenol or toluene or degrade TCE (strains G4 5223 and 5231) were found to revert to phenol utilization through the constitutive expression of Tom ($PR1_{23}$, and $PR1_{31}$). These strains were found to be genetically stable through at least 100 generations of nonselective growth and, without induction, capable of expressing Tom and catechol-2,3-dioxygenase at 50-100% of the wild type fully induced levels (9), and consequently degrade TCE without exogenous aromatic inducers. Such strains are attractive as biodegradative agents for the remediation of groundwater contaminated with TCE.   Neither $PR1_{23}$ or $PR1_{31}$ contain recombinant DNA sequences. Manipulations were through genetic techniques normally encountered in the environment. The only "foreign" DNA is that of Tn5 , a transposon known to exist in the environment, and is under no known barrier for natural transfer to strains like G4.

5.   Genetics

The Tom operon has been shown to be encoded by a newly described toluene degradative plasmid completely unrelated to the archetypical TOL (10) called TOM. A linkage map of TOM_{31c} is shown in Figure 1. In a previous study we successfully transferred this plasmid to two strains selected for their potential to form biofilms: *Pseudomonas* sp. JS150 and

SmaI

BamHI

NheI

KpnI

TOM₃₁C

TOM_{31C}

114
kb

H

H

J

I

Region of Tn5 homology

tomA

Region of Tn5 homology

0

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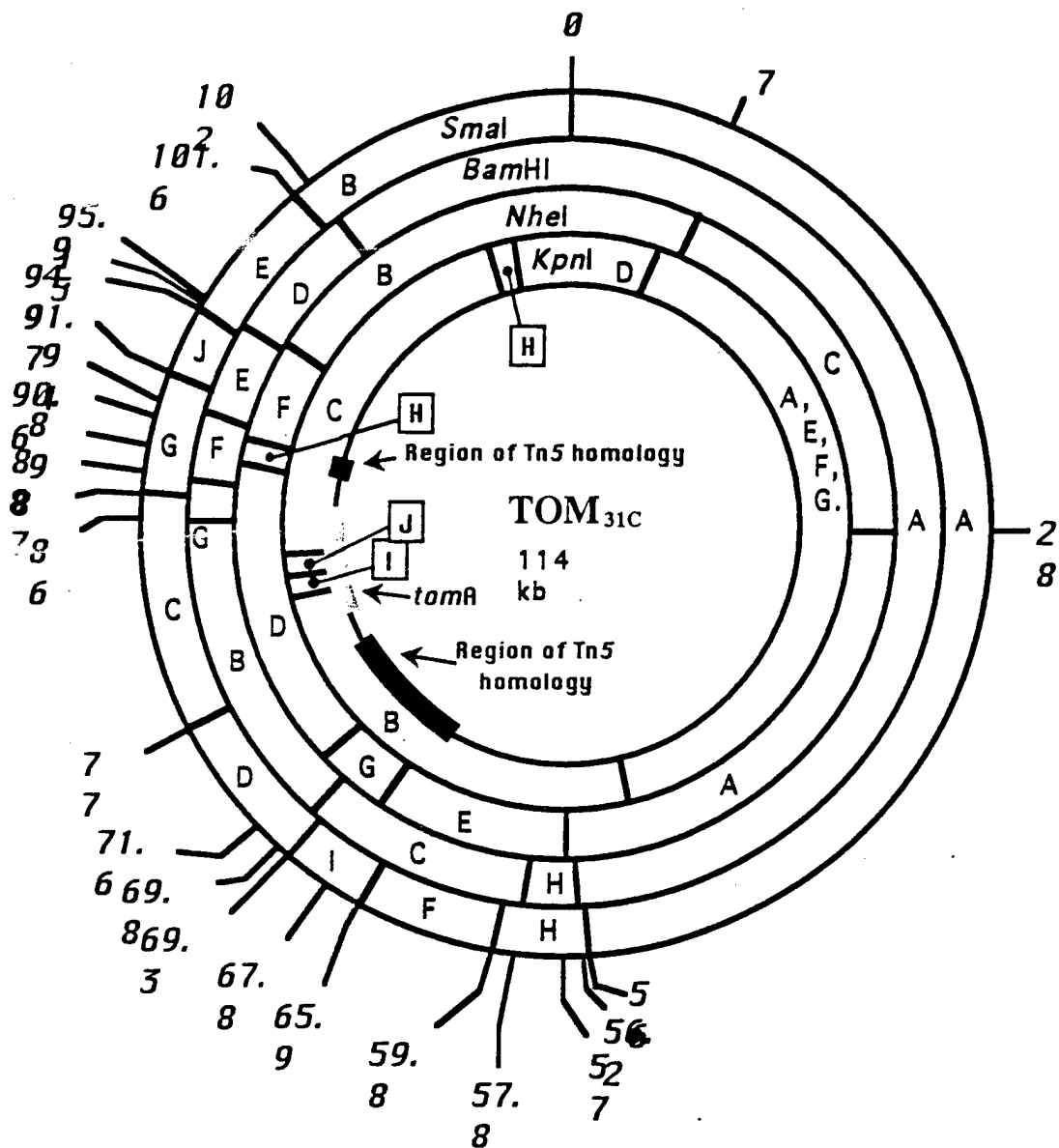
Figure 1. Restriction Linkage Map of TOM₃₁c

Burkholderia cepacia 249, that consequently also expressed Tom, constitutively. This readily demonstrated the capacity for transfer of this plasmid between strains, and the stable constitutive expression of Tom in alternate hosts.

C.   SCOPE

The research outlined in this report has one primary objective and one secondary. The primary objective is to convert naturally occurring organisms (bacteria) found at TCE-contaminated groundwater sites, to constitutive TCE degraders. The secondary objective is to evaluate these strains (once produced) under laboratory simulations of environmental conditions for TCE degradation, selectability, and survival.

The experimental approach to this first task has been divided into two general areas: (1) Bacterial Enrichment to find bacterial candidates to receive the constitutive genes and, (2) Transfer of $pTOM_{31c}$ to these Enrichment Isolates.

> Phase I: <u>Isolation of putative FAV host strains</u>. This phase involved the isolation of bacteria from various soil and aquifer samples based on their ability to rise to dominance following a carbon amendment of the material. Our objective was to isolate strains that would become dominant when introduced to a contaminated soil or aquifer environment, along with a selective carbon and energy source. The carbon sources selected for these isolations were glucose, lactate, IGEPAL, and ethanol.

> Phase II: <u>TOM31c transfer to FAV strains and screening for constitutive Tom expression</u>. After they were isolated the FAV strains were mated with bacteria carrying TOM_{31c}. This particular plasmid was chosen because it has been mapped and is known to contain Tn*5* thus allowing an easier isolation of TOM transconjugants by virtue of encoded kanamycin resistance.

> Phase III: <u>Effectiveness of Tom constitutive FAV strains in native soil and groundwater from West Florida</u>. The capacity for these strains to degrade TCE under field conditions was always the accepted target of this research. To determine this potential the various FAV-TOM_{31c} transconjugants were added to TCE-contaminated native soil and water samples along with the carbon sources used for their selection. Their capacity to · degrade TCE was measured.

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ISOLATION OF PUTATIVE FAV HOST STRAINS

A.   INTRODUCTION

Bacteria were isolated from various soil and aquifer samples following enrichment with several carbon sources. These carbon sources were chosen based on their potential utility as carbon and energy sources that might be added to a contaminated aquifer during an *in situ* bioaugmentation treatment for the degradation of chloroethenes. Our objective was to isolate strains that rose to dominance during such treatments. Such strains would then be subjects for use in Section III, where the transfer of constitutive TCE degradative capabilities would be attempted.

The first step is to enrich for bacteria likely to be useful for reapplication to a contaminated aquifer. These bacteria are being isolated based on key physiological characteristics judged "exploitable." The parameters represent two practical themes of control upon their release. Bacteria were selected that are already dominant, or that rise to dominance in the field samples following amendment with glucose, lactate, ethanol or IGEPAL. This is because there is a strong likelihood that such isolates could easily be encouraged to have a dominant role at their (or similar) site of isolation during TCE treatment. The other classes of bacteria under enrichment are those that could utilize a particularly unusual or strongly selective agent or carbon source. To some extent, we thought the ethanol degraders would fit into this category, but ethanol seems to be selective only due to toxic effects at concentrations in water above 2 percent. This is not a practical target in view of oxygen demands:

$$\text{Ethanol Oxidation: } C_2H_6O + 3\ O_2 \longrightarrow 2\ CO_2 + 3\ H_2O$$
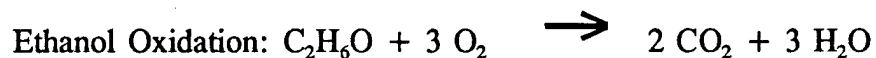
This relationship describes a minimal oxygen demand of 3:1 (O_2 :ethanol) on a molar basis. 2% ethanol ~ 20 g/L, or (@ 46 g/m) is approximately 0.43 M. Therefore water treated to this concentration would require = 1.3 moles O_2 per liter of water (3 x 0.43 moles) treated. This means supplying 41.6 grams of O_2 per liter (O_2 = 32 g/mole) just to supply the oxidation needs of the primary substrate! There must be a surplus of O_2 if the monooxygenase that actually carries out the initial transformation of TCE is to act. Since this level is hardly

attainable, our goal is to concentrate on substrates that can be utilized within reasonable ranges of oxygen demand ($\leq$ 100 mg O_2 per liter). This means that using carbon sources based on their selective capacity through toxic effects is not promising. Peroxide (H_2O_2) can be a source of oxygen. However, peroxide is highly toxic to bacteria and therefore highly selective. This was incorporated as one of the selective agents for two reasons. One is of course the selectivity, and the second is because of its frequent choice as an alternative method for introduction of O_2 to groundwater.

$$2H_2O_2 \rightarrow O_2 + 2H_2O$$

The concept of using selective agents to encourage select soil bacterial populations has also occurred to others. Lajoie *et al.* have reported the screening of various detergents in this role for the development of what they term "Field Application Vectors" (FAVs) (5, 6). The detergent IGEPAL CO-720 is a nontoxic, water soluble, non-ionic substrate that allowed the selection of bacteria which subsequently dominated IGEPAL-amended soils.

B. MATERIALS AND METHODS

1. Organisms and Culture Conditions

Bacteria isolated from the various carbon source enrichments were not characterized beyond their Gram reaction, antibiotic sensitivities and capacity to utilize several specific carbon sources such as phenol and toluene.

2. Media

Basal salts medium (BSM) (4) was used and contains the following components at pH 7.2 (in g/l water): $K_2HPO_4 \cdot 3H_2O$ (4.25), $NaH_2PO_4 \cdot H_2O$ (1), NH_4Cl (2), Disodium salt of nitrilotriacetic acid: [$HO_2CCH_2N(CH_2CO_2Na)_2$] (0.12), $MgSO_4 \cdot 7H_2O$ (0.2), $FeSO_4 \cdot 7H_2O$ (0.012), $ZnSO_4 \cdot 7H_2O$ (0.003), and $MnSO_4 \cdot H_2O$ (0.003). BSM concentrations and concentrations of various components were altered as described for various aspects of this project.

An artificial groundwater medium (AGM) was also employed during the carbon source phase, since it was considered a more representative chemical environment than the

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bacteria would likely encounter during a bioaugmentation approach. AGM consisted of (in g/l water): $NaHCO_3$ (0.17)), $MgSO_4$ (0.03), KCl (0.004), $CaCl_2 2 H_2O$ (0.004), $NaSiO_3 \cdot 9 H_2O$ (0.014), $(NH_4)_2HPO_4$ (0.26), NH_4NO_3 (0.16), NH_4Cl (0.05), $CaSO_4 2 H_2O$ (0.005), Ethylenediamine- tetraacetic acid, disodium salt dihydrate (0.006), $Na_2B_4O_7 10 H_2O$ (0.004), $FeCl_2 4 H_2O$ (0.001), $MnSO_4 H_2O$ (0.0008), $CoCl_2 6 H_2O$ (0.00002), Na_2MoO_4 (0.00005), $ZnSO_4 7 H_2O$ (0.00003).

3. Antibiotic Resistance

To identify enriched strains of bacteria once reintroduced, it was reasoned that those bacteria that could be enumerated by a specific nutrient media: R2A could be typed according to antibiotic resistance patterns. Such a "fingerprint" would offer a way to characterize isolates with a reasonably large portion of their cellular physiology (because antibiotics can be selected for diverse sites of action: including cell wall anabolism, protein synthesis and DNA synthesis inhibitors). The specific zones of resistance to as many as six antibiotics can be determined for a given strain on a standard plate, in a 24-hour period using paper discs impregnated with a carefully regulated dose of antibiotics, applied to a newly seeded lawn of bacterial isolate.

The pattern of antibiotic sensitivity was determined by uniformly spreading the organism on LB plates made of identical volumes (25 mL). Discs containing the antibiotics were dropped onto the plates. The bacteria were allowed to grow overnight, and the clear zone around each disc was measured from the edge of the disc to the limit of the zone. The following antibiotics were used (amount per disk is given in parentheses): Ampicillin (Ap) (10 μg), Chloramphenicol (Cm) (30 μg), Kanamycin (Km) (30 μg), Nalidixic acid (Nal) (30 μg), Piperacillin (Pip)(100 μg), Rifampin (Rif) (5 μg), Streptomycin (Sm) (10 μg), Tetracycline (Tc) (30 μg), Trimethoprim (Tmp) (5 μg), and Triple Sulfa (SSS) (300 μg).

4. Carbon Source Utilization Profiles

Isolates representing potential FAVs were screened for selectable traits encoded by TOM_{31c}, including the utilization of phenol (2mM), toluene (vapor fed), and *ortho*-cresol (0.5 mM) as sole carbon sources, as well as the enrichment substrates ethanol (20 mM),

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phthalate (20 mM), and lactate (20 mM) in BSM-purified agar.

To test for growth at 42°C and under anaerobic conditions, the bacteria were plated on media designed for heterotrophic organisms from aqueous environments (R2A media) and placed in the proper environment. This was done to determine if high temperature growth could be used in the genetic selection procedure for transconjugants following matings.

C.   RESULTS AND DISCUSSION

1.   Bacterial Enrichment

Six samples (collected from various contaminated aquifers) were available for enrichment studies. Enrichment cultures were begun and serial dilution plates made from the different samples to isolate dominant native organisms from the aquifers. Samples were labeled according to the site from which they were obtained (Table 1):

Table 1. AQUIFER ABBREVIATIONS AND DESCRIPTIONS

Designation	Samples	Aquifer Location	Contaminant
NF1476	well water	Whiting Field, N.A.S. (North Field, Monitoring well # unknown)	Diesel
MW07A,B,D	well water	Whiting Field, N.A.S. (South Field, Monitoring wells	TCE and BTEX
WFB	well water	Whiting Field, N.A.S. (South Field, Monitoring well # unknown)	TCE
UW	well water & sediment	Pensacola, U.W.F.[1]	none
EP	well water	Pensacola, E.C.U.A.[1] (East plant)	TCE
MF	sediment	Moffett Field, N.A.S.	TCE
KA	well water	Gilbert Mosely Site, Witchita, KS	TCE
WS	sediment	Gilbert Mosely Site, Witchita, KS	TCE
BR	sediment	Borden Aquifer, Canada	none

[1] Escambia County Utilities Authority (E.C.U.A.)

a. Glucose, Lactate and Ethanol Enrichment Cultures from MWO7A and MWO7D

Twelve sterile 125 mL shake flasks each contained a 20 mL sample of the contaminated ground water. Chemicals were added to the samples to produce a 0.1x concentration of BSM. Each set of four shake flasks received: 20 mM glucose, 20 mM lactate, or 3% ethanol.

Different amounts of H_2O_2 were added to the enrichments so that each carbon source included a control with no H_2O_2, and three different H_2O_2 concentrations of 100 mg/L, 500 mg/L, and 1000 mg/L. The enrichments were incubated at room temperature on a shaker for 120 hours.

Isolation. Serial dilutions in sterile 1x BSM were prepared from the enrichment cultures and plated on the enrichments carbon source. Dominant colony types were picked from the plates and streaked for isolation prepared for phase II characterization.

b. Glucose, lactate and ethanol enrichment cultures from MWO7B and NF1476

The enrichment procedure used for MWO7A and MWO7D samples was also employed here.

Isolation. All the serial dilutions were plated on R2A agar in addition to the corresponding enrichments carbon source. Serial dilutions were also prepared directly from the ground water and plated on R2A agar, 20 mM glucose, 20 mM lactate and 3% ethanol.

Dominant colonies were chosen for phase II characterization.

c. Glucose, lactate and ethanol enrichment cultures from WA

Five grams of soil were mixed with 6 mL of sterile 1x BSM and vortexed for approximately 5 minutes. Twelve sterile shake flasks were prepared for the enrichments by adding sterile 1x BSM and carbon to produce a 10:1:5 C:N:P ratio. Each set of four flasks received a different carbon source of either 20 mM glucose, 20 mM lactate, or 20 mM ethanol.

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Different amounts of H_2O_2 were added so that each carbon enrichment contained 0, 100 and 500 mg/L H_2O_2. Five mL of the soil-BSM mixture were added to each flask and incubated at room temperature on a shaker for 120 hours.

Isolation. Serial dilutions were prepared directly from the soil-BSM mixture, H_2O_2 enrichment cultures after 72 hours of incubation, and all the enrichment cultures after 120 hours of incubation. All serial dilutions were plated on R2A agar and the enrichments carbon source. Dominant colonies were prepared for phase II characterization.

d. Glucose, Lactate, Ethanol and IGEPAL Enrichment Cultures from UW

Ten liters of groundwater were passed through a 0.2 μm filter. The filter was placed into a sterile test tube filled with 6 mL of 1x BSM and vortexed for approximately 5 minutes. The vortexed solution was then used to inoculate the enrichment flasks. The same enrichment procedure was used as described for WA samples.

One liter of UWF groundwater was amended with IGEPAL (0.1% wt/wt) in 169 ppm NH_4Cl and 42 ppm K_2HPO_4 and allowed to shake at room temperature for 2 weeks.

Isolation. Serial dilutions in BSM were prepared from the enrichment cultures. The dilutions were plated on R2A agar and 0.1% IGEPAL agar. Dominant colonies were prepared for partial characterization.

Isolation. The same isolation and characterization procedures were used as described for WA samples.

Bacterial populations following lactate, glucose and ethanol enrichments from NF, MW07B, UW, and WA are depicted in Figures 2, 3, and 4. Respectively, these point to several conclusions: Most of the organisms grew best on R2A agar. Many H_2O_2 enrichment cultures showed no growth when plated on defined carbon plates; however, when plated on R2A, a significant number of cells were enumerated. The 20 mM ethanol, 100 mg/l H_2O_2, WA enrichment, however, did grow exceptionally well on 20 mM ethanol, yielding more cells than the similar enrichment lacking H_2O_2 (Figure 4). This indicated that H_2O_2 was of limited use in selecting specific hydrocarbon utilizing populations. With the exception of the high-concentration (20 mM) ethanol utilizers, H_2O_2 was toxic at the concentrations employed. Better growth was obtained with just the ambient oxygen available in the sealed tubes.

e. Glucose, Lactate, Ethanol and IGEPAL Enrichment Cultures from WFB,
    KA and EP

Five hundred mL of groundwater was passed through a 0.2 μm filter and vortexed with 6 mL of 1 x BSM for approximately 5 minutes. Twelve sterile shake flasks were prepared for the enrichments by adding 1 x BSM and carbon to produce a 10:1:5 C:N:P ration. Each set of four flasks received a different carbon source of either 20mM glucose, 20 mM lactate , 20 mM ethanol or 1% IGEPAL in 169 ppm NH_4Cl and 42 ppm K_2HPO_4 instead of 1 x BSM. Different amounts of peroxide were added so each enrichment contained either 0mg/L, 100mg/L, or 500 mg/L H_2O_2. The 1 x BSM vortexed solution was then used to inoculate the enrichment flasks which were incubated at room temperature on a shaker for 120 hours.

Isolation. Serial dilutions in 1x BSM were prepared from the enrichment cultures after 72 hours of incubation, and again after 120 hours. The dilutions were plated on R2A agar in addition to the corresponding enrichments carbon source. Serial dilutions were also prepared directly from the groundwater and plated on R2A agar, 20mM glucose, 20 mM lactate, 20mM ethanol, and 1% IGEPAL. Dominant colonies were prepared for characterization.

f. Glucose, lactate, ethanol and IGEPAL enrichment cultures from MF and
    WS.

Five grams of soil were mixed with 6 mL of 1 x BSM and vortexed for approximately 5 minutes. The vortexed mixture was then used to inoculate the enrichment flasks. The same procedure for enrichment cultures and isolation was used as for the WFB, KA and EP samples.

An IGEPAL enrichment was done using 5 grams of Moffet Field sediment in 45 mL of distilled water. The same enrichment and isolation procedures described for the UW material were followed.

Populations monitored by growth on R2A medium following the glucose, lactate, ethanol and IGEPAL (with and without H_2O_2) enrichments of EP, WS, KA, MF, and WF are depicted in Figures 5-12.

12
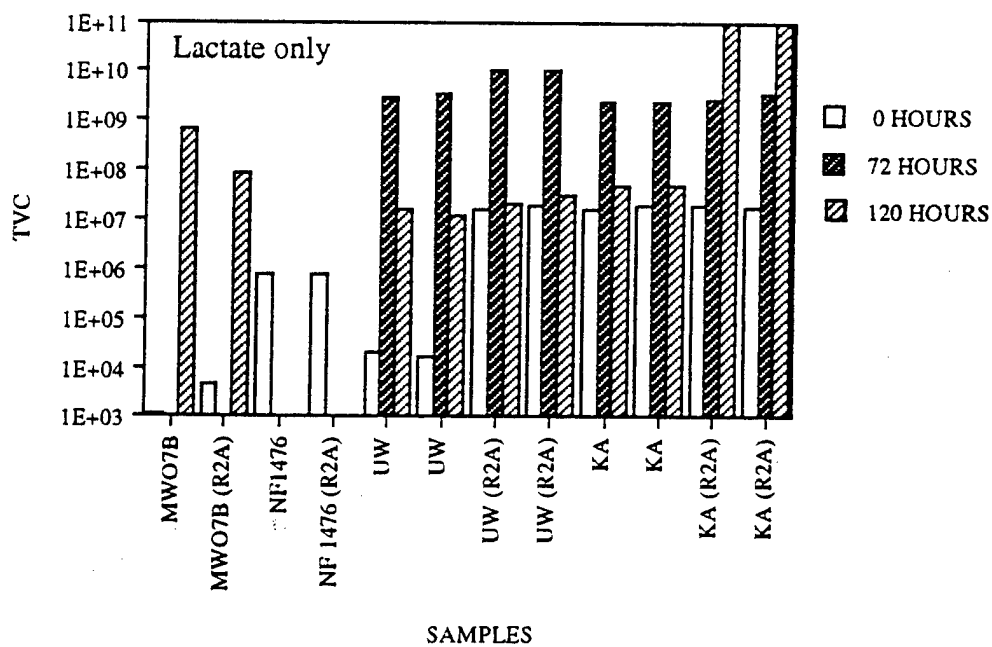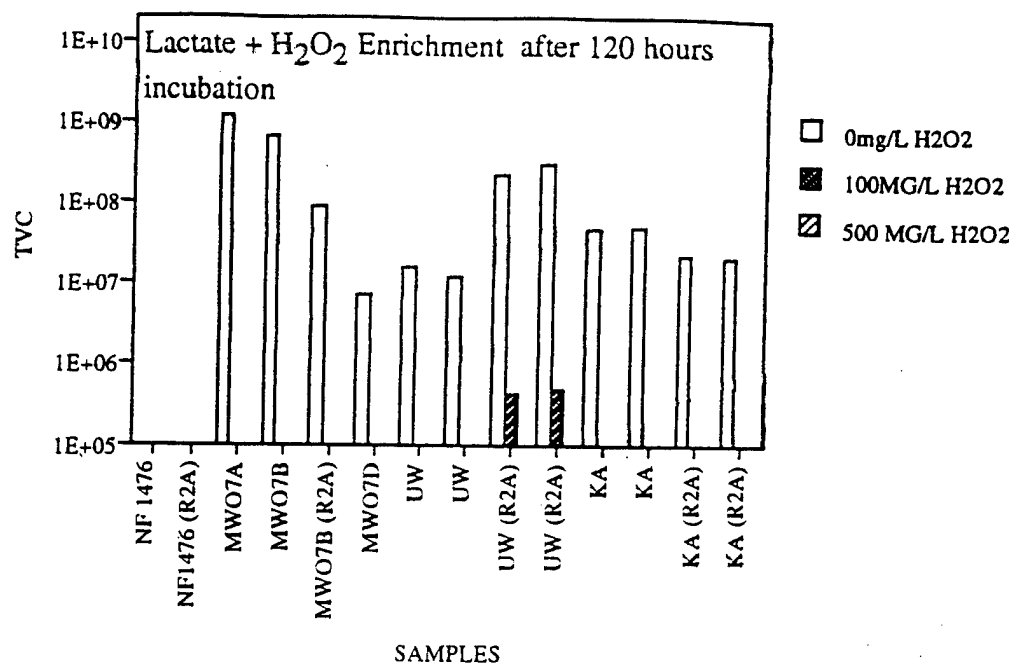
Figure 2. Lactate Enrichments

13
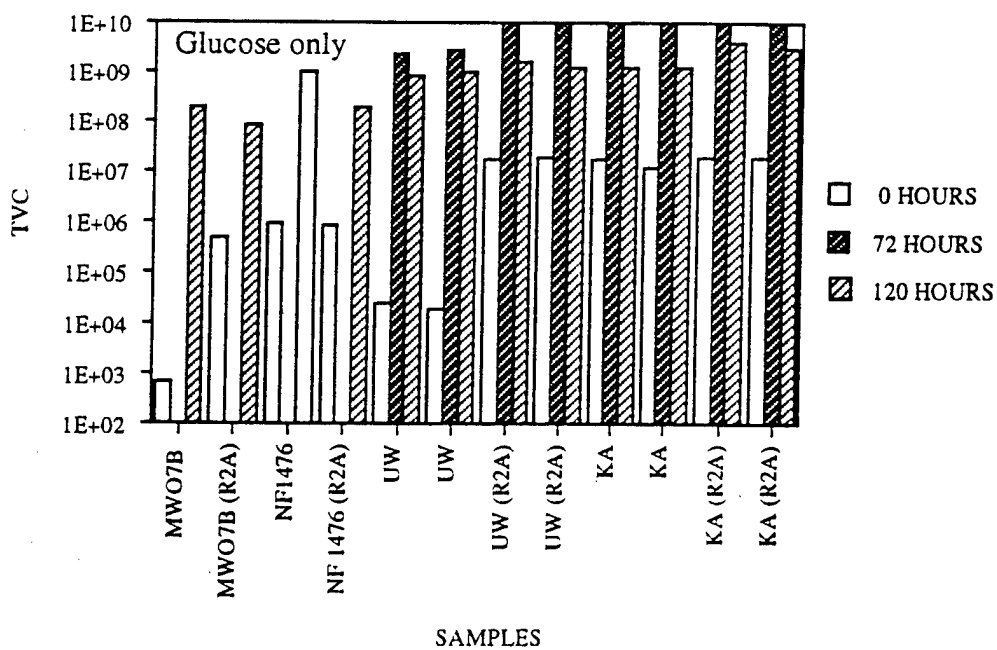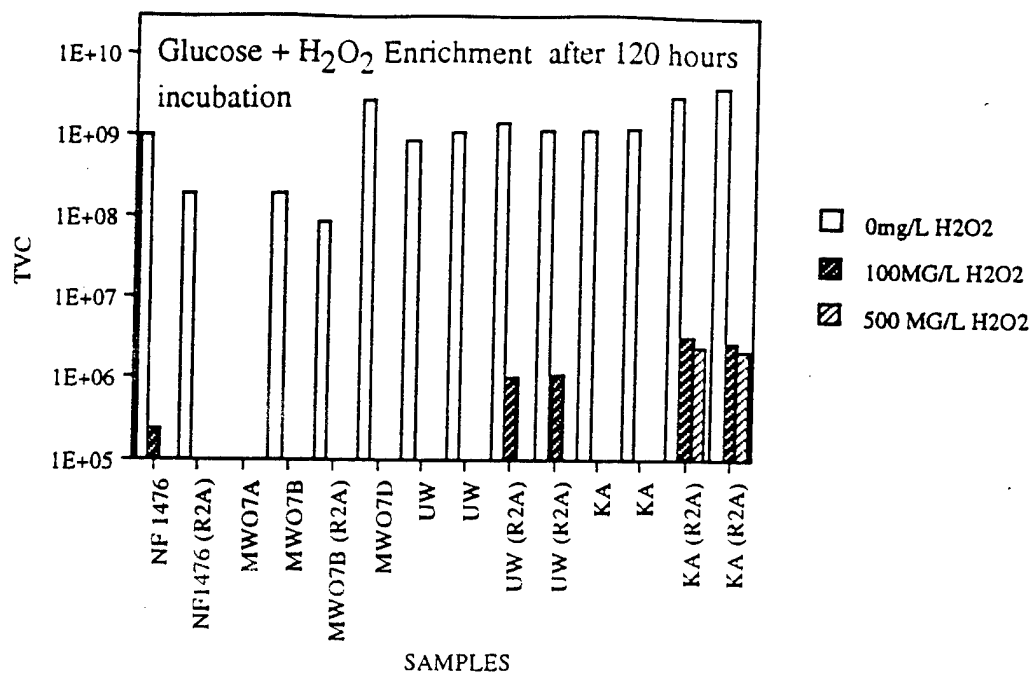
Fig 3. Glucose Enrichments.
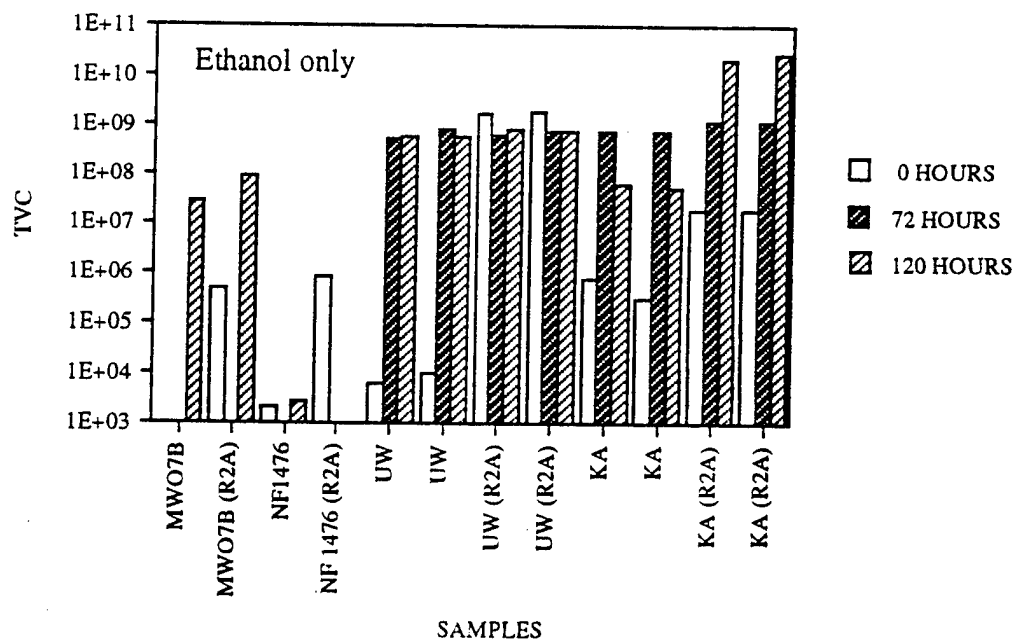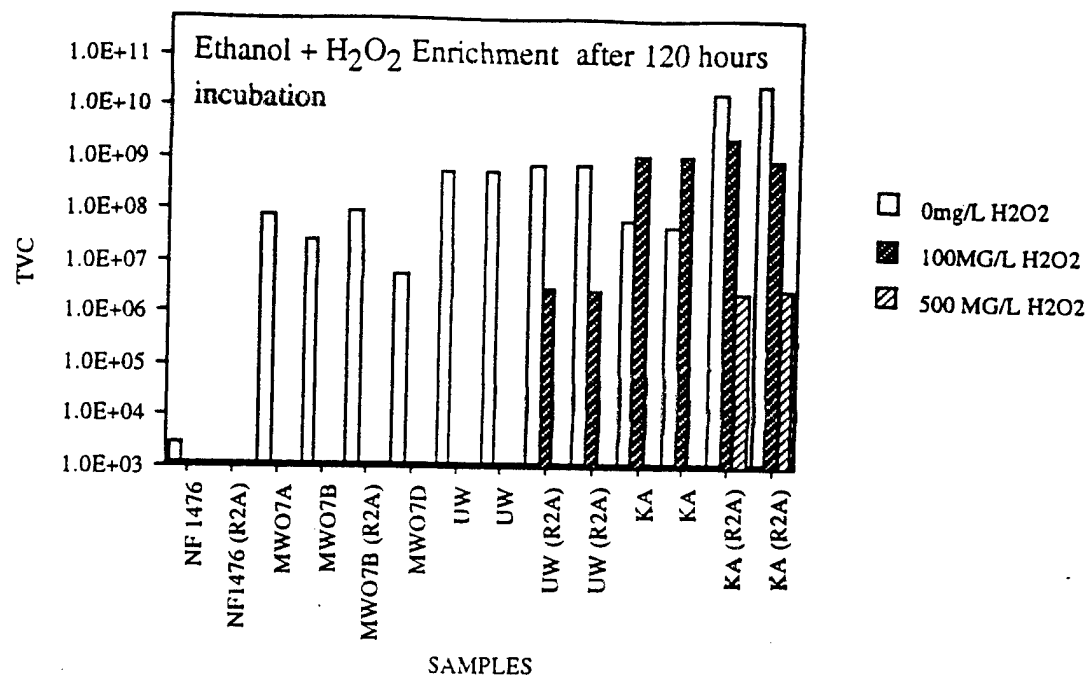
Fig 4. Ethanol Enrichments.

For most of the organisms, 500 mg/L H_2O_2 was lethal; however, the organisms in samples WFB, MF, and WS showed a significant amount of growth in 100 mg/L and 500 mg/L H_2O_2 when utilizing 20 mM glucose, thus indicating that for these glucose utilizing populations the H_2O_2 (while not toxic) was not of any obvious benefit within this test system.

None of the organisms were able to survive peroxide addition to the 1% IGEPAL enrichments. In fact, the IGEPAL enrichments displayed the lowest TVCs when compared to the other carbon enrichments.

The new IGEPAL degraders were partially characterized and the results are depicted in Tables 2 and 3. One Moffet Field isolate and three from UWF aquifer water were obtained.
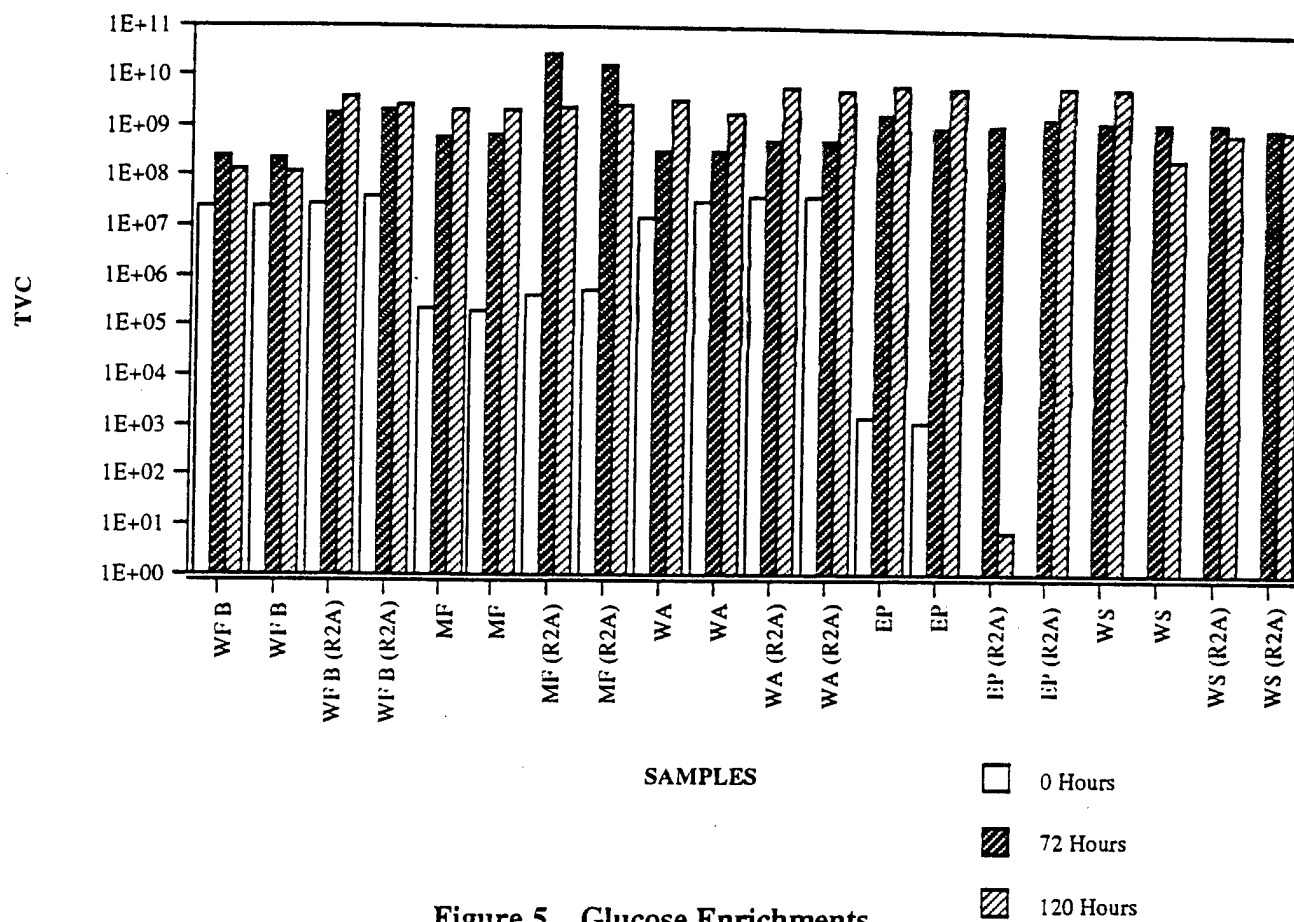
Figure 5 . Glucose Enrichments
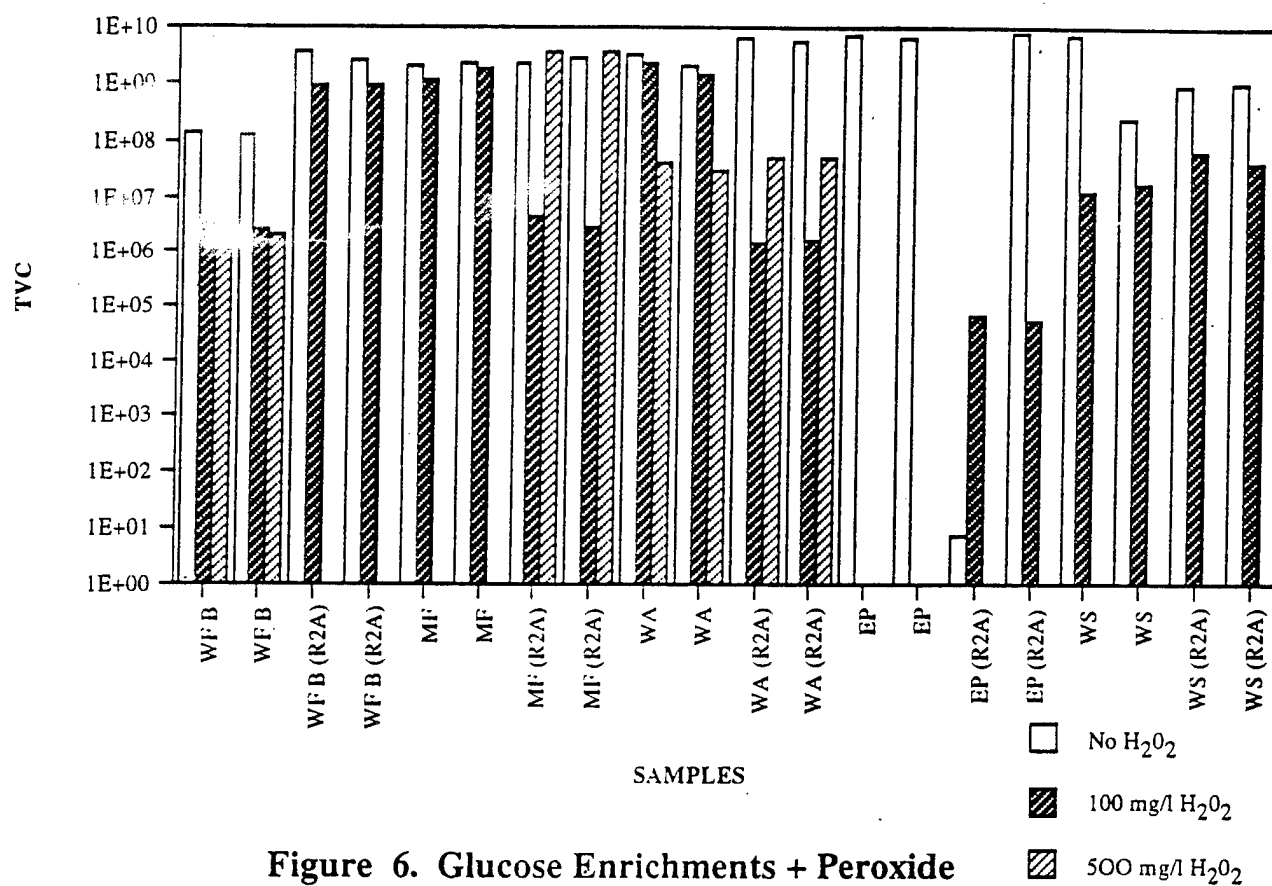
Figure 6. Glucose Enrichments + Peroxide

Legend:
- ☐ No H_2O_2
- ▨ 100 mg/l H_2O_2
- ▨ 500 mg/l H_2O_2

Y-axis: TVC (values from 1E+00 to 1E+10)

X-axis: SAMPLES

Sample labels: WF B, WF B, WF B (R2A), WF B (R2A), MF, MF, MF (R2A), MF (R2A), WA, WA, WA (R2A), WA (R2A), EP, EP, EP (R2A), EP (R2A), WS, WS, WS (R2A), WS (R2A)
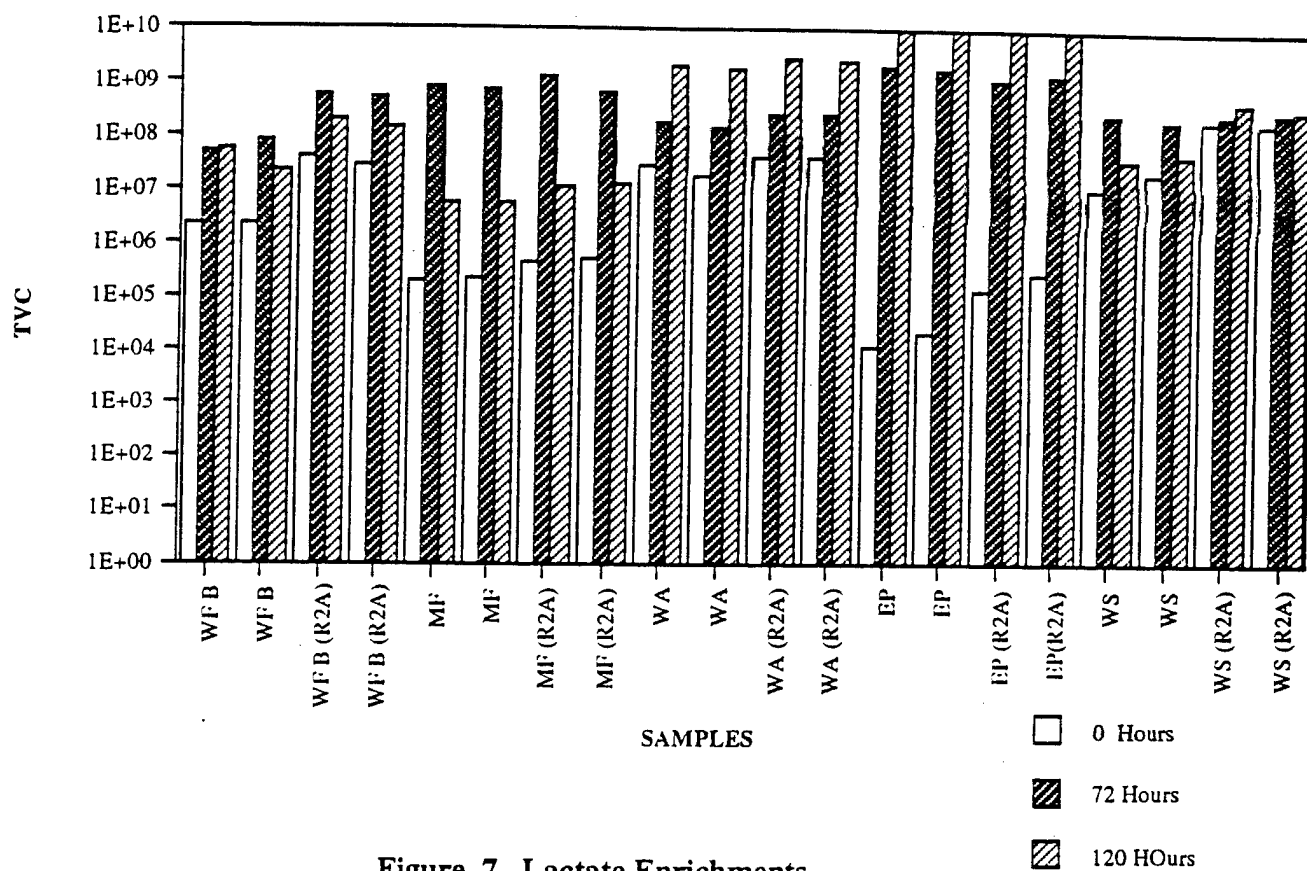
Figure 7. Lactate Enrichments

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TVC

1E+10
1E+09
1E+08
1E+07
1E+06
1E+05
1E+04
1E+03
1E+02
1E+01
1E+00

WF B | WF B | WF B (R2A) | WF B (R2A) | MF | MF | MF (R2A) | MF (R2A) | WA | WA | WA (R2A) | WA (R2A) | EP | EP | EP (R2A) | EP(R2A) | WS | WS | WS (R2A) | WS (R2A)

SAMPLES

☐ No H_2O_2

▨ 100 mg/l H_2O_2

▨ 500 mg/l H_2O_2
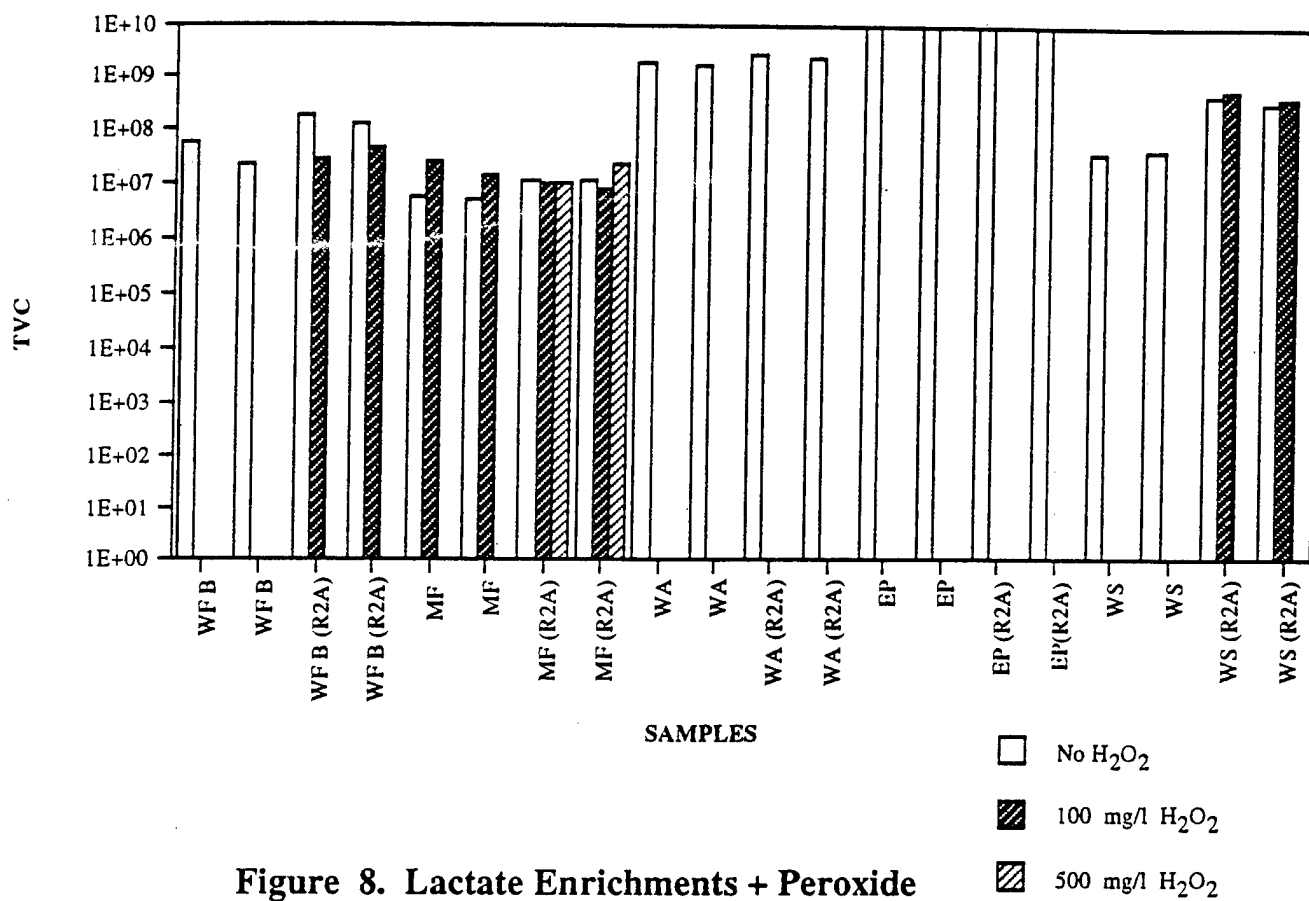
Figure 8. Lactate Enrichments + Peroxide

20
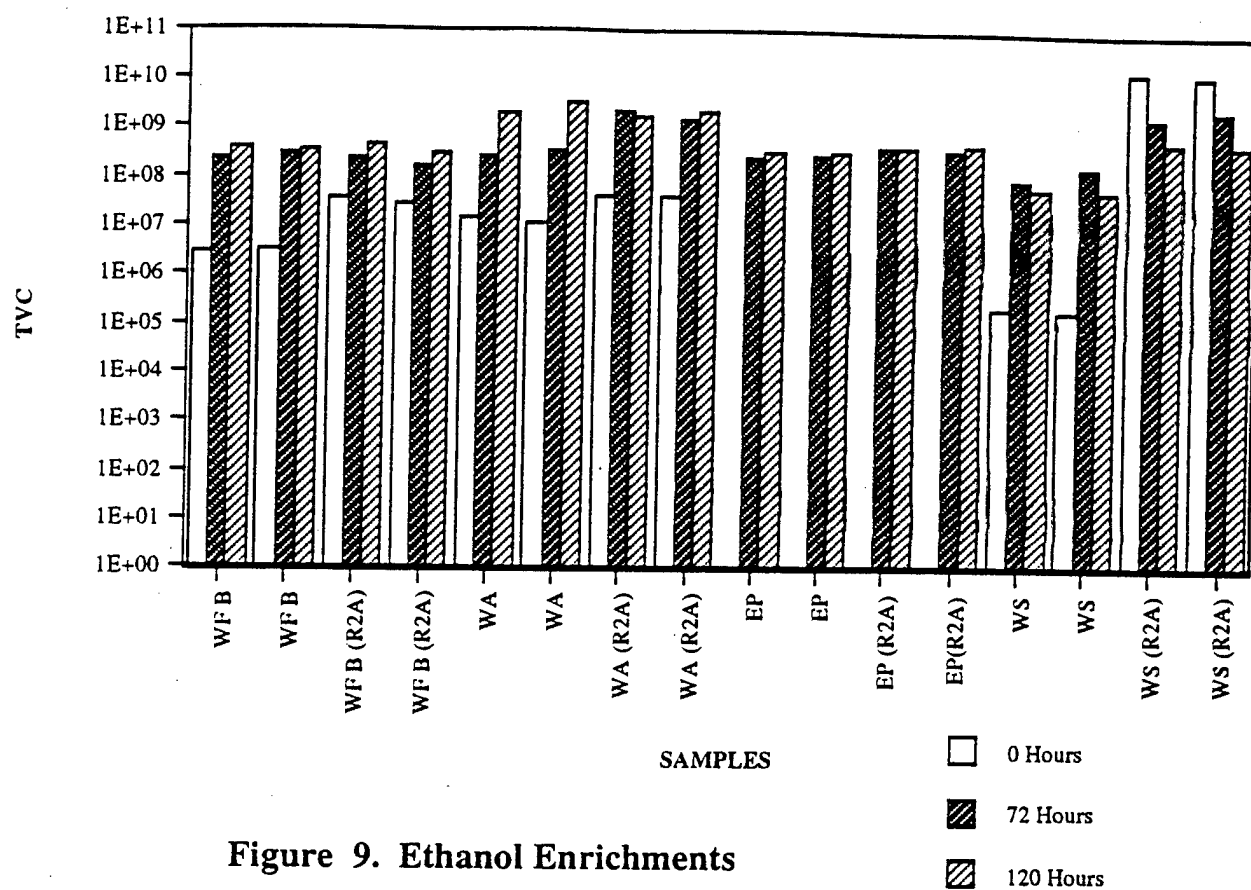
Figure 9. Ethanol Enrichments

Legend:
- ☐ 0 Hours
- ▨ 72 Hours
- ▨ 120 Hours

Y-axis: TVC
X-axis: SAMPLES

X-axis labels: WF B, WF B, WF B (R2A), WF B (R2A), WA, WA, WA (R2A), WA (R2A), EP, EP, EP (R2A), EP(R2A), WS, WS, WS (R2A), WS (R2A)

Y-axis values: 1E+00, 1E+01, 1E+02, 1E+03, 1E+04, 1E+05, 1E+06, 1E+07, 1E+08, 1E+09, 1E+10, 1E+11
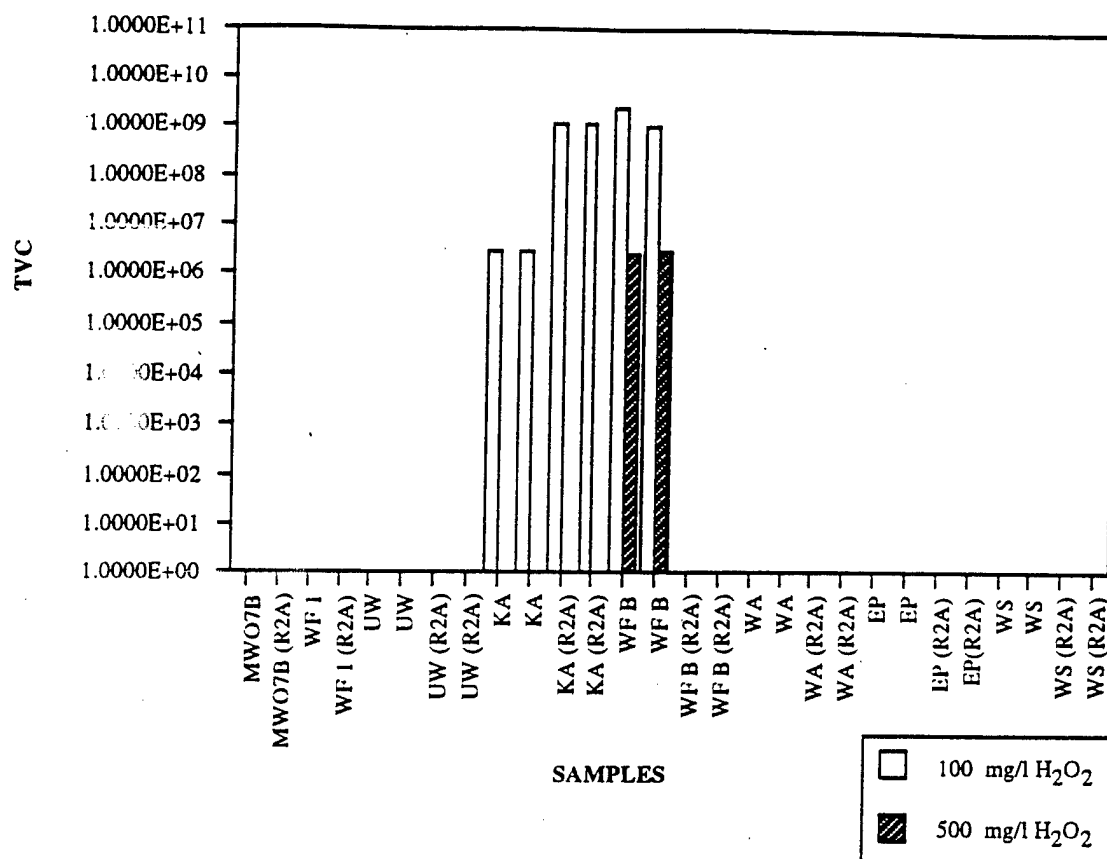
Figure 10. Ethanol Enrichments + Peroxide

22
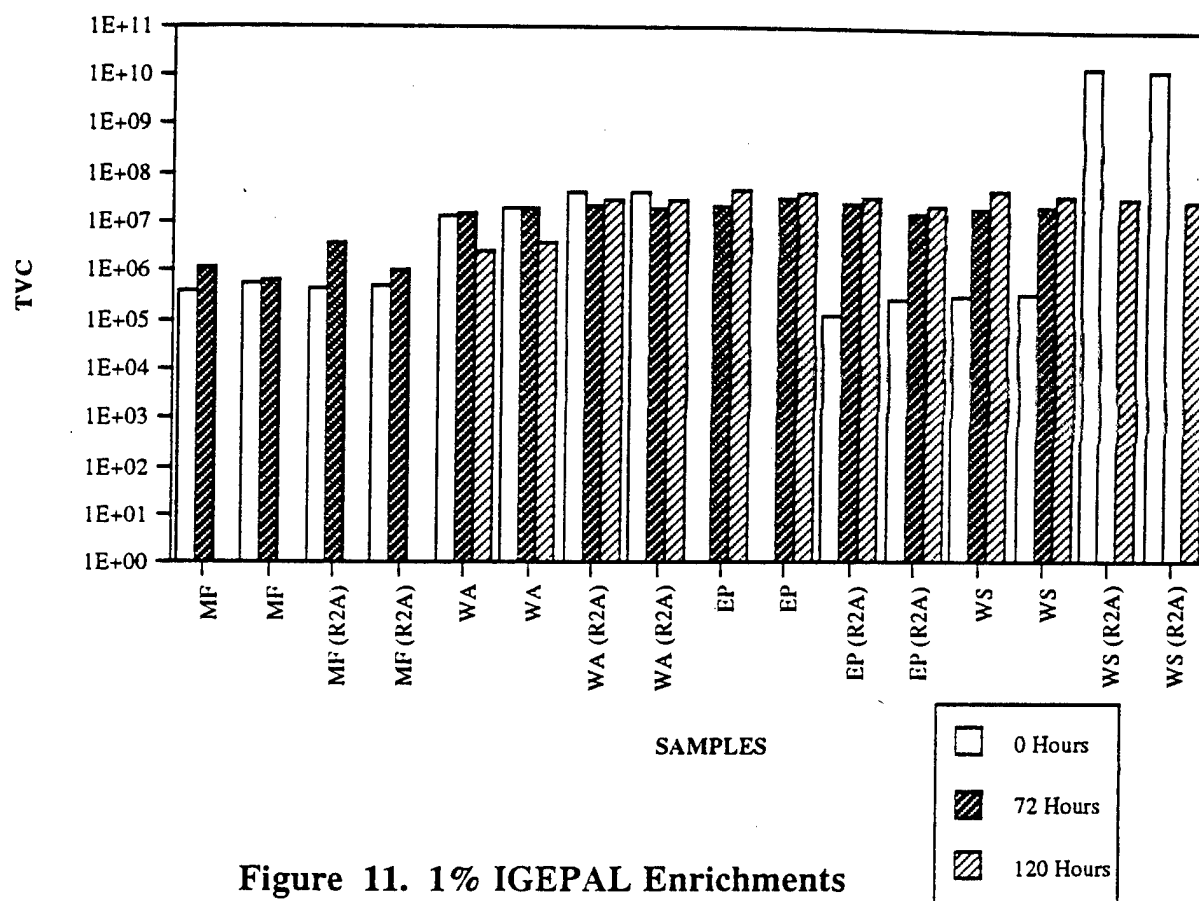
Figure 11. 1% IGEPAL Enrichments

Chart axes and labels:

Y-axis: TVC — 1E+11, 1E+10, 1E+09, 1E+08, 1E+07, 1E+06, 1E+05, 1E+04, 1E+03, 1E+02, 1E+01, 1E+00

X-axis: SAMPLES — MF, MF, MF (R2A), MF (R2A), WA, WA, WA (R2A), WA (R2A), EP, EP, EP (R2A), EP (R2A), WS, WS, WS (R2A), WS (R2A)

Legend:
- 0 Hours
- 72 Hours
- 120 Hours

23
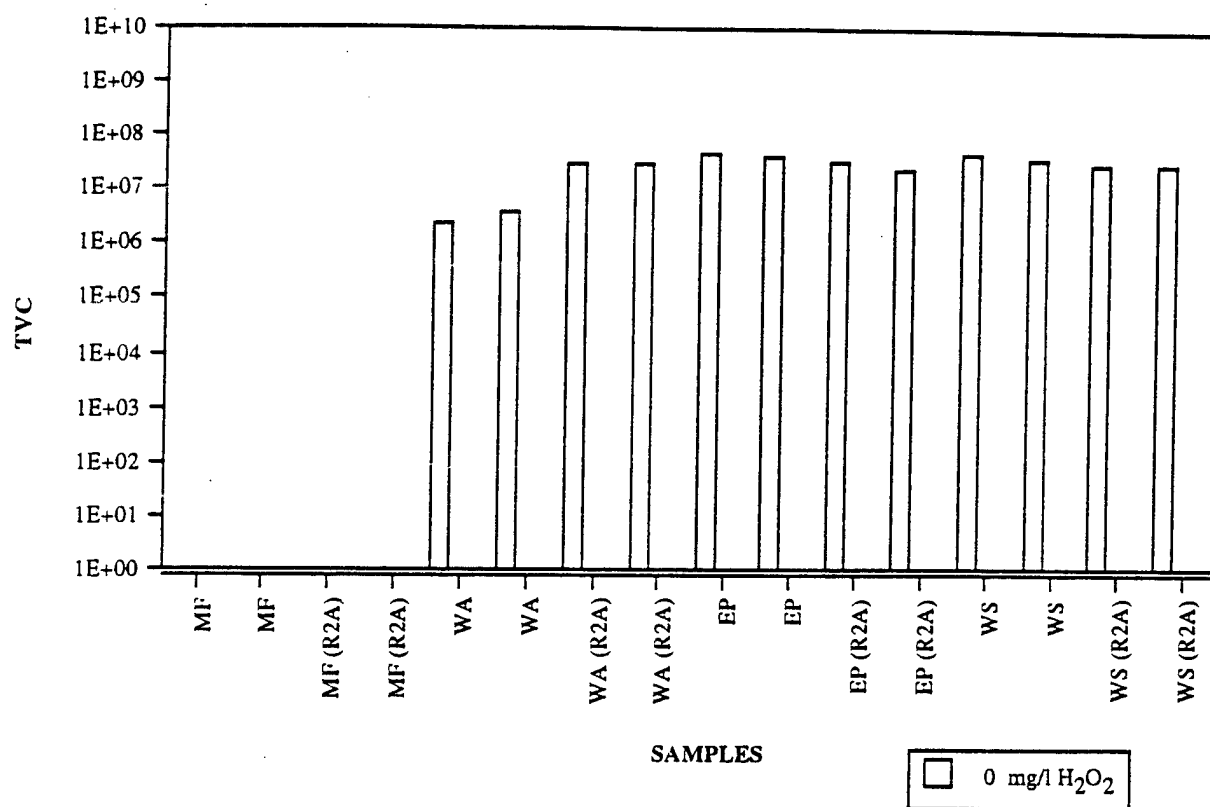
Figure 12. 1% Igepal Enrichments + Peroxide

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Table 2. PARTIAL CHARACTERIZATION OF IGEPAL DEGRADERS

Strain	Gram	Colony morph.	Toluene	O-cresol	ethanol	Phenol	Phthalate	Lactate	42°
MFI-1	-	off white	(-/-)	(-/-)	(-/-)	(-/-)	(-/-)	(+/+)	(-/-)
UWI-1	-	yellow	(-/-)	(-/-)	(-/-)	(-/-)	(-/-)	(-/-)	(-/-)
UWI-2	-	pink	(-/-)	(-/-)	(+/+)	(-/-)	(-/-)	(+/+)	(-/-)
UWI-3	-	off white	(-/-)	(-/-)	(-/-)	(-/-)	(-/-)	(-/-)	(+/+)

Table 3. ANTIBIOTIC RESISTANCE PATTERNS OF IGEPAL DEGRADERS

Strain	AM	C	K	NA	PIP	RA	S	TE	TMP	SSS
MFI-1	0	5	8	0	10	2.5	3.5	0	0	8
UWI-1	2	11	4.5	10.5	0	13.5	0	11	1	0
UWI-2	0	0	8	0	15	11.5	3.5	9.5	0	0
UWI-3		16	16	12.1	0	.5			0	

2.   Screening of Enrichment Isolates as Prospective Recipients of TOM_{31c}

To select transconjugants of TOM_{31c} from Escherichia *coli* (TOM_{31c}) or *Pseudomonas cepacia* (TOM_{31c}) donors, it was first necessary to know what antibiotic counterselections were possible. Table 4 contains the results from the preliminary antibiotic sensitivity characterization of the groundwater isolates.

Table 4. ANTIBIOTIC RESISTANCE PROFILE[1]

STRAIN	Ap	Cm	Km	Nal	Pip	Rif	Sm	Tc	Tmp	SSS
249-2	5.5	7 nc	0	5	11		0	2	8 nc	0
A2-1	0	2	6.5	1.5	6	2.5	3	5	0	5
BR-3	3	13	0	17			0	14		
BR-4	7	6	1.5	0		8	1	4	0	0
BR-5	7.5 nc	12	7 nc	10	4.5	9	2 nc	11	2	0
BR-5 (TOM$_{31c}$)	10.5	10	0	10			0	7		
BR-7	0	9.5	0	4	4.5	18	0.5 nc	10	15	8
BR-8	1		1	4	4.5		2	10		
BR-9	11	2	4	0	10	15	3.5	7		0
BR-10	10	11	0.5	0	8	13.5	4.5	9.5	8.5	1 nc
BR-11	0	4 nc	1	0.5	5.5	15	1	2	4	0
BR-13	0	2	3	1	6 nc	2	4	3	1.5	3 nc
BR-14	0	0.5	2	1	4	1	5	2.5	0	3.5 nc
BR-18	0	0	8	1.5	8	0.5	4	4	0	4.5
WS-19	0	0	6	0.5	5		3	1		
WS-21	2.5	5	9	0	6	1	8	5	0	3 nc
WS-22	0	0	10	3			6	8 nc		
WS-23	7.5	12.5	1 nc	12	8.5	7.5	4	10	0	6
WS-25	7	5.5	7 nc	6.5	4	1	0.5	6	0.5	12 nc
BR-26	0	10	4 nc	10			1	12		
BR-27	0	7	4	8	19	0	3	7	0	0.5
EP-1	7	3	10		7	5	3	3	7 nc	6 nc
EP-2	3	10 nc	1.5 nc	10	6	10	2 nc	7	0	2 nc
EP-2(T)	3	12 nc	0	7	5	0	3	4	0	0
EPE-1	0	0	0	0	0	0	0	0	0	0
EPE-2	0	1.5	0	6	5 nc	2	5	2.5	0	6
EPI-1	0	5 nc	0	5	0	3.5 nc	0	1	0	5 nc
EPI-1(T)	0	5.5 nc	0	5	0	3	0	2	0	0
EPI-2	3 nc	2.5	4	3	0	2		3	1	0

Table 4. ANTIBIOTIC RESISTANCE PROFILE[1] (continued)

STRAIN	Ap	Cm	Km	Nal	Pip	Rif	Sm	Tc	Tmp	SSS
EPL-1	0	1 nc	1	4.5	5 nc	2	6	2	0	2 nc
EPL-2	10 nc	5	5 nc	3	10	10 nc	3	5	0	
KAE-1	0	1.5	1	7	7	1.5	6	5	0	5.5
KAE-2	2	7 nc	0	5.5	7	1.5 nc	1.5	3.5	8 nc	0
KAI-3	0	0.5	1.5	3	5	0.5	6	3.5	0	6 nc
MF-A	0	3	2	5	1	0.5	5 nc	2	0	0
MF-B	0	1	5 nc	5	5	2.5	3	3	0	8
MFE-2	0	5	4	4	5	0	4	3	7.5 nc	0
MFE-4	2.5	2	1	2 nc	11	2.5	0	4	0	
MFG-1	0	0.5	9	2.5	8	2 nc	6	4	0	0
MFG-2	0		7	1.5 nc	1	5	3	0	0	
MFG-2 (T)	2 nc	10 nc	0	10	16	2	0	5	5	0
MFG-3	0	0.5	6	1 nc	7.5	1	3	3	0	0
MFG-4	0	2 nc	8	1 nc	8	0.5	5.5	3	0	0
MFI-1	0	5	8	0	10	2.5	3.5	0	0	8
MFL-1	0	1.5 nc	6	5	1	5.5 nc	2.5	0	5	
MFL-2	0	1	1.5	7	5.5 nc	1	5.5	3.5	0	5 nc
MKPNL9cr	0	0	8	0	6	0	6 nc	0	0	4 nc
NF-1	0	1	2.5	1	5 nc	6	2	3.5	3 nc	0
NF-2	0.5		0	6	5	0	1	4	0	7 nc
NFE-1	0	0	0	6.5	5	2	5	3	0	10 nc
NFE-2	1	0	5.5	6	5	1 nc	5	4	0	
NFE-3	0.5	0	5.5 nc	7	6	2 nc	5 nc	4	0	3.5 nc
NFG-1	0	2	8 nc	4	5	2	4 nc	5	0	2 nc
NFG-2	0	2	7 nc	4	11 nc		2 nc	6		
NFG-3	2.5	2	1.5	5.5	5	1.5	0	8	0	7.5 nc
NFG-4	4	2	0.5	9		6.5 nc	0	3	0	0
NFL-1	0	0.5	0	6	3.5	2.5	4	4.5	0	9 nc
NFL-2	0	1.5	2.5	7	6	2	1	4	0	5 nc

Table 4. ANTIBIOTIC RESISTANCE PROFILE[1] (continued)

STRAIN	Ap	Cm	Km	Nal	Pip	Rif	Sm	Tc	Tmp	SSS
NFL-3	0.5	0	5	5	4.5	1	4	3	0	0
PR1	0	2.5	0	6.5	6	3	5.5	3.5	0.5	0
UWI-1	2	11	4.5	10.5	0	13.5	0	11	1	0
UWI-2	0	0	8	0	15	11.5	3.5	9.5	0	0
UWI-3		16	16	12.1	0	0.5			0	
UWL-1	7	4	5	6.5	6	2.5	1.5	5	0	8
UWL-2					0	0			0	0
Vitreocella	0	2 nc	7.5	0	8	1	0	0	0	0
KA-1	0	0.5	7	2	8	1	3	3	0	3 nc
KA-2	0	0	6.5	2	5	1	5	2	0	2.5 nc
KA-2(T)	0	5.5	0	4	0	1	0	2	0	0
KA-3	0	0	6 nc	2.5 nc	12 nc	1	4.5	1.5	0	2 nc
KAG-1	1 nc	8	0	5	7.5 nc	1 nc	1.5	4	0	0
KAG-2	0	0	0	0	0	0	0	0	0	0
KAG-3	9	4	3	2 nc	7	6	1	4	0	1.5 nc
WF-GATT-A	2	8	0	6	6	0	1.5	6	0	0
WF-GATT-B	0	6	0	4	0	3	0	1.5	0	6 nc
WF-GATT-B(T)	0.5 nc	5.5	0	5	6 nc	1	4	6	0	0
WFA-1	0	1	1.5	0	3	2	5	2	0	10 nc
FA-4	0	10 nc	0	5	6.5	0	0	2	0	7 nc
WFB-1	0	11	0	11	3	8	0	7	0	0
WFB-2	0	1.5	1	6.5	6.5	2	6	4	0	6
WFBE-1	0	1.5	0	6	8 nc	7.5	0	2	9	0
WFBE-2	0	1	1	7	5.5	2	6.5	4.5	0	5
WFBE-3	0	1	0	7	7 nc	7	0	2.5	10	0
WFBG-1	2.5 nc	8	0		8	0.5 nc	2 nc	5	7 nc	0
WFBG-2	0	7.5	0	6	5	8	0	6.5	10	0
WFBI-1	14	9	7	4	14	13	4	8	8 nc	0
WFBI-2	12.5	9.5	7	4.5	12	10	2	9	0	0

Table 4. ANTIBIOTIC RESISTANCE PROFILE[1] (concluded)

STRAIN	Ap	Cm	Km	Nal	Pip	Rif	Sm	Tc	Tmp	SSS
WFBL-1	0	5	0	5 nc	0	0	0	2 nc	2 nc	10 nc
WFBL-2	0	2	1	7	6.5	2	6.5	4	0	5
WFBL-3	0	1.5	1.5	6.5	7	2	6.5	4	0	6
WFBL-4	0	6	0	5 nc	0	0	0	2 nc	0	9 nc
WFC	0	4	5.5	5.5	7	0	0	4	8 nc	0
WSE-1	0	2 nc	0	5.5	5	2	6	2	0	7
WSE-2	0	1 nc	0	6	5.5 nc	2	6	2	0	6
WSG-1	0	1.5 nc	6	1.5	6 nc	1	5	2	0	0
WSG-2	1	9	0	10	3.5	5.5	0.5 nc	3	0	0
WSG-3	0	0	0	0	0	0	0	0	0	0
WSI-1	2.5 nc	3	7	3	5	1.5	4	4	0	2 nc
WSL-1	0	0	7	2 nc	7	1	4	3	1.5 nc	2

[1] Zones of Antibiotic Sensitivity are measures as the zone of growth in mm from the edge of the disc. 0 mm indicates complete resistance to that antibiotic.

SECTION III

TOM$_{31c}$ TRANSFER TO FAV STRAINS AND SCREENING FOR THE CONSTITUTIVE EXPRESSION OF Tom

A. INTRODUCTION

After they were isolated, the FAV strains were mated with bacteria carrying TOM$_{31c}$. This particular plasmid was chosen because it has been mapped and is known to contain Tn5 thus allowing an easier isolation of TOM transconjugants by virtue of encoded kanamycin resistance (Km^r). Since we focused on TOM$_{31c}$, it was necessary to limit potential client organisms to gram negatives, isolated from the aforementioned materials and enrichments. Characterizations were performed for carbon source utilization (toluene, o-cresol, ethanol, phenol, phthalate, and lactate), growth at high temperature (42°C), growth under facultative anaerobic conditions, and antibiotic resistance patterns (to: Ampicillin (Amp), Chloramphenicol (Cm), Kanamycin (Km), Nalidixic Acid (Nal), Piperacillin (Pip), Rifampin (Rif), Streptomycin (Str), Tetracycline (Tc), Trimethoprim (Tmp), and Triple Sulfa (SSS)).

Attempts were made to transfer the plasmid TOM$_{31c}$, which encodes for Km^r, several carbon source markers, and constitutive cometabolic degradation of trichloroethylene, to these putative FAVs through conjugational matings with *Escherichia coli* (TOM$_{31c}$) and *B. cepacia* (TOM$_{31c}$), electroporation and biolistic transformation.

To create a strain capable of persisting in an aquifer environment and constitutively cometabolizing trichloroethylene (TCE), an attempt will be made to transfer the plasmid TOM$_{31c}$ from laboratory strains (*Escherichia coli* HB101 and *B. cepacia* 249-2) into the native environmental microorganisms via the process of conjugation. Eventually, other strains containing the degradative plasmid (such as *B. cepacia* 17616 and PR1) may be used in an attempt to gain additional transconjugants.

Since environmental regulations governing the release of bacteria containing PR1$_{31c}$ are in question, another constitutive version of the TOM plasmid was considered for investigation in this experiment. Strain PR1$_{301}$ containing TOM$_{301c}$ was created through chemical mutagenesis and is therefore free of the environmental regulation constraints encountered for TOM$_{31c}$ because it contains Tn5. The presence of Tn5 was the reason for choosing TOM$_{31c}$ for the primary

experiments since it also provides unique Tn5 hybridization sequences in addition to the positive selection phenotype provided by the Km^r gene.

B.   MATERIALS AND METHODS

1.   Organisms and Culture Conditions

Burkholderia cepacia G4-PR1$_{31}$ was developed in this laboratory following Tn5 mutagenesis of G4 followed by biochemical characterization and selection for spontaneous loss of the mutant phenotype (10). *B.cepacia* G4-PR1$_{301}$ was isolated as the product of nitrosoguanidine mutagenesis, and Pip counterselection with phenol. The enriched mutants unable to degrade phenol were identified on minimal medium supplemented with phenol, yeast extract and the reducing indicator dye, triphenyltetrazolium chloride, as previously described for Tn5 mutants (10). The degradative plasmid responsible for the constitutive expression of the TCE degrading enzyme Tom in this strain is designated TOM$_{301c}$. HB101 is a auxotrophic strain of *E. coli* utilized because of its capacity for transformation and lack of cross hybridization with gene probes used. Strains used in this study are listed in Table 5. Other strains were environmental isolates characterized in Section I. Growth of all organisms was routinely accomplished on BSM medium supplemented with lactate, phenol or one of the selective carbon sources developed in Section I.

Table 5. BACTERIAL STRAINS AND PLASMIDS

Strains	Description[2]	Reference
PR1$_{31}$ (TOM$_{31c}$)	*Km^r, Phe$^+$, C23O$^+$, Tom^c, phenol utilizing revertant of G4 5231 (TOM$_{31}$::Tn5)*	9
PR1$_{301}$ (TOM$_{301c}$)	Km^s, Phe$^+$, C23O$^+$, Tom^c, phenol utilizing revertant of G4$_{301}$ (TOM$_{301}$)	This article
Plasmids		
TOM$_{31c}$	Tn5 induced mutant of the B. *cepacia* G4 plasmid TOM that gave spontaneously became constitutive for the expression of Tom	9
TOM$_{301c}$	Nitrosoguanidine induced mutant of the B. *cepacia* G4 plasmid TOM that gave spontaneously became constitutive for the expression of Tom	Unpublished work
pGEM4Z	Ap^r E. *coli* cloning vector	Promega Laboratories Madison, WI
pMS64	An 11 kb EcoRI clone from TOM$_{31c}$ containing *tom*A and B genes inserted into pGEM4Z	10

Abbreviations: phe$^+$, capable of phenol utilization; phe$^-$, incapable of phenol utilization, phe^r, revertible to phenol utilization; Tom^{i,c}, inducible ^{i}, or constitutive ^{c} expression; Km^r, Ap^r, resistance to kanamycin, or ampicillin.

2.   TCE Degradation Analyses

        TCE degradation analyses were carried out from either liquid samples extracted with pentane or gas headspace samples analyzed on a Hewlitt-Packard 5890 Gas chromatograph using a VOCOL column and either an electron capture or photoionization detector as previously described (11, 3).

TCE pentane extractions of the putative FAVs and 249-2 (TOM_{31c}) were performed in order to determine their capacity to express Tom constitutively following overnight growth in R2A broth. A 5 mL sample of each culture was put into a 15 mL vial, sealed with Teflon-lined septa and crimped shut. TCE was added to produce a concentration of approximately 500 ug/L. A negative control containing only R2A and TCE was prepared in addition to the cultures.

The vials were allowed to shake at 30°C for 24 hours before adding 5 mL of pentane. They were then shaken for an additional 2 hours to ensure TCE extraction. The pentane was withdrawn and run on the gas chromatograph (GC) . These experiments were performed in triplicate.

An air headspace TCE degradation assay of the putative FAVs and $PR1_{31c}$ were conducted to determine the relative rate and extent of TCE degradation. The organisms were grown overnight in 20 mL of R2A broth, pelleted, and resuspended in 1x BSM. A 5 mL sample of each resuspended culture was put into 15 mL vials and sealed. TCE was introduced at 800 ug/L-1000 ug/L. A negative control consisted of only 1x BSM and TCE.

The vials were then inverted and allowed to shake at room temperature for 90 minutes; Gas headspace samples were analyzed via GC at 15-minute intervals. These determinations were performed in triplicate.

3.   Conjugation.

Five μL of overnight cultures of the donor and each recipient were plated on Luria Broth medium. Five μL of the donor and each recipient were mixed in a third spot on the same LB plate. The plates were incubated overnight, and cells from each sample were transferred by toothpick to a selective plate and grown overnight. Alternatively, 5 mL of each of the overnight cultures were mixed, filtered to a 0.45 μm filter, and the whole filter was aseptically transferred to the LB plate and incubated overnight. After overnight growth and mating the cells are harvested from the membrane surface by vortexing in 1x BSM which was then plated to selective medium.

Two mating schemes were followed to encourage conjugation and obtain putative transconjugants of those strains of interest. During one phase of the work, only FAVs which were Phe⁻ and Km⁵ were considered as target recipients of the plasmid TOM_{31c} whereas at other times randomly selected available environmental isolates that were Phe⁻Km⁵ with the biolistic

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device. This includes only those that were isolated from Glucose, Lactate, Ethanol, or IGEPAL enrichments. In addition, previously, only HB101 (TOM$_{31c}$), 249-2 (TOM$_{31c}$), and PR131(TOM$_{31c}$) were used as potential donors. During the second phase, transconjugants which had been obtained during an earlier phase were also considered and used as potential donors of (TOM$_{31c}$). This includes MFG-2 (TOM$_{31c}$), MF-4 (TOM$_{31c}$), BR-5 (TOM$_{31c}$), and WS-23 (TOM$_{31c}$).

Using the antibiotic profiles of each of the FAVs which were Gram -, Phe⁻, and Km^a, appropriate double antibiotic selections were chosen to be used in the conjugal matings. Table 6 shows all the possible selections for each of the potential donor and recipient pairs.

Initially, simple spot matings on agar surfaces were conducted. If a putative transconjugant was not obtained using this protocol, it as repeated using a filter mating technique provided by S. Enfinger. This method employs mixing potential donors and recipients together in a liquid phase and then passing them through a sterile 0.2-μm Nylon filter using Swinnex filter holders and sterile 3- or 5-cc syringes.

Table 6. TRANSCONJUGATIONAL STRATEGIES TESTED

		Selection	
Donor	Recipient	Donor/Recipient	Growth[1]
249-2 (TOM$_{31c}$)	**BR-5**	**No lysine/Km**	**+**
249-2 (TOM$_{31c}$)	BR-8	No lysine/Phenol	-
249-2 (TOM$_{31c}$)	BR-3	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	BR-10	No lysine/Phenol	-
249-2 (TOM$_{31c}$)	BR-17	No lysine/Phenol	-
249-2 (TOM$_{31c}$)	**WS-23**	**No lysine/Phenol**	**+**
249-2 (TOM$_{31c}$)	MF-A	No lysine/Phenol	-
249-2 (TOM$_{31c}$)	MF-B	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	WFC	No lysine/Phenol	-
249-2 (TOM$_{31c}$)	NF-1	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	MFE-2	No lysine/o-cresol	-
249-2 (TOM$_{31c}$)	**MF-4**	**No lysine/Phenol**	**+**
249-2 (TOM$_{31c}$)	NFL-1	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	NFE-2	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	WF-GATT-A	No lysine/Toluene	-

Table 6. TRANSCONJUGATIONAL STRATEGIES TESTED
(Continued)

Donor	Recipient	Selection Donor/Recipient	Growth[1]
249-2 (TOM$_{31c}$)	WF-GATT-B	No lysine/Phenol	+
249-2 (TOM$_{31c}$)	BR-14	No lysine/Toluene	-
HB101 (TOM$_{31c}$)	BR-8	Phenol/Phenol	-
HB101 (TOM$_{31c}$)	BR-10	Phenol/Phenol	-
HB101 (TOM$_{31c}$)	BR-17	Phenol/Phenol	-
HB101 (TOM$_{31c}$)	WS-21	Phenol/Phenol	-
HB101 (TOM$_{31c}$)	MF-A	Phenol/Phenol	-
HB101 (TOM$_{31c}$)	MF-B	Toluene/Toluene	-
HB101 (TOM$_{31c}$)	WFC	Phenol/Phenol	-
HB101 (TOM$_{31c}$)	NF-1	Toluene/Toluene	-
HB101 (TOM$_{31c}$)	MFE-2	Toluene/Toluene	-
HB101 (TOM$_{31c}$)	MF-4	Phenol/Phenol	-
HB101 (TOM$_{31c}$)	NFL-1	Toluene/Toluene	-
HB101 (TOM$_{31c}$)	NFE-2	Toluene/Toluene	-
HB101 (TOM$_{31c}$)	WF-GATT-A	Toluene/Toluene	-
HB101 (TOM$_{31c}$)	WF-GATT-B	Phenol/Phenol	-
HB101 (TOM$_{31c}$)	BR-14	Toluene/Toluene	-
HB101 (TOM$_{31c}$)	BR-3	Toluene/Toluene	-
PR1 (TOM$_{31c}$)	BR-10	Nalidixic/Phenol	-
PR1 (TOM$_{31c}$)	BR-17	Nalidixic/Phenol	-
PR1 (TOM$_{31c}$)	WS-21	Nalidixic/Phenol	-
PR1 (TOM$_{31c}$)	MF-4	Nalidixic/Phenol	-
249-2 (TOM$_{31c}$)	BR-3	No lysine/o-cresol	-
249-2 (TOM$_{31c}$)	BR-8	No lysine/Phenol	-
249-2 (TOM$_{31c}$)	BR-10	No lysine/Phenol	-
249-2 (TOM$_{31c}$)	BR-14	No lysine/o-cresol	-

Table 6. TRANSCONJUGATIONAL STRATEGIES TESTED
(Continued)

Donor	Recipient	Selection Donor/Recipient	Growth[1]
249-2 (TOM$_{31c}$)	BR-17	No lysine/Phenol	-
249-2 (TOM$_{31c}$)	EPI-1	No lysine/Phenol	+
249-2 (TOM$_{31c}$)	WS-21	No lysine/Phenol	-
249-2 (TOM$_{31c}$)	MF-A	No lysine/Phenol	-
249-2 (TOM$_{31c}$)	MF-B	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	WFC	No lysine/Phenol	-
249-2 (TOM$_{31c}$)	NF-1	No lysine/o-cresol	-
249-2 (TOM$_{31c}$)	MFE-2	No lysine/o-cresol	-
249-2 (TOM$_{31c}$)	NFL-1	No lysine/o-cresol	-
249-2 (TOM$_{31c}$)	NFE-2	No lysine/o-cresol	-
249-2 (TOM$_{31c}$)	WF-GATT-A	No lysine/o-cresol	-
249-2 (TOM$_{31c}$)	WF-GATT-B	No lysine/Phenol	-
249-2 (TOM$_{31c}$)	UWL-1	No lysine/o-cresol	-
249-2 (TOM$_{31c}$)	KAE-1	No lysine/Toluene	-
PR1 31 (TOM$_{31c}$)	BR-3	Anaerobic/o-cresol	-
PR1 31 (TOM$_{31c}$)	BR-8	Anaerobic/Phenol	-
PR1 31 (TOM$_{31c}$)	BR-10	Anaerobic/Phenol	-
PR1 31 (TOM$_{31c}$)	BR-14	Anaerobic/o-cresol	-
PR1 31 (TOM$_{31c}$)	BR-17	Anaerobic/Phenol	-
PR1 31 (TOM$_{31c}$)	WS-21	Anaerobic/Phenol	-
PR1 31 (TOM$_{31c}$)	MF-A	42C/Phenol	-
PR1 31 (TOM$_{31c}$)	MF-B	Anaerobic/Then toluene-	
PR1 31 (TOM$_{31c}$)	WFC	42C/Phenol	-
PR1 31 (TOM$_{31c}$)	NF-1	Anaerobic/o-cresol	-
PR1 31 (TOM$_{31c}$)	MFE-2	42C/o-cresol	-
PR1 31 (TOM$_{31c}$)	MF-4	Anaerobic/Phenol	-

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Table 6. TRANSCONJUGATIONAL STRATEGIES TESTED
(continued)

Donor	Recipient	Selection Donor/Recipient	Growth[1]
PR1 31 (TOM$_{31c}$)	NFL-1	Anaerobic/o-cresol	-
PR1 31 (TOM$_{31c}$)	NFE-2	42C/o-cresol	-
PR1 31 (TOM$_{31c}$)	WF-GATT-A42C/o-cresol		-
PR1 31 (TOM$_{31c}$)	WF-GATT-B42C/Phenol		-
PR1 31 (TOM$_{31c}$)	UWL-1	Anaerobic/o-cresol	-
PR1 31 (TOM$_{31c}$)	KAE-1	Anaerobic/Then toluene	-
249-2 (TOM$_{31c}$)	EP-2	No lysine/Phenol	+
249-2 (TOM$_{31c}$)	WA-2	No lysine/Phenol	+
249-2 (TOM$_{31c}$)	WFBI-1	No lysine/Phenol	-
249-2 (TOM$_{31c}$)	WFBI-2	No lysine/Phenol	-
249-2 (TOM$_{31c}$)	MFG-1	No lysine/Phenol	-
249-2 (TOM$_{31c}$)	**MFG-2**	**No lysine/Phenol**	**+**
249-2 (TOM$_{31c}$)	MFG-3	No lysine/Phenol	-
HB101 (TOM$_{31c}$)	MF-4	Phenol/Phenol	-
249-2 (TOM$_{31c}$)	MFG-4	No lysine/Phenol	-
249-2 (TOM$_{31c}$)	MFL-1	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	MFL-2	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	WFBL-2	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	WFBL-3	No lysine/Toluene	-
HB101 (TOM$_{31c}$)	WFBI-1	Phenol/Phenol	-
HB101 (TOM$_{31c}$)	WFBI-2	Phenol/Phenol	-
HB101 (TOM$_{31c}$)	MFG-1	Phenol/Phenol	-
HB101 (TOM$_{31c}$)	MFG-2	Phenol/Phenol	-
HB101 (TOM$_{31c}$)	MFG-3	Phenol/Phenol	-
HB101 (TOM$_{31c}$)	MFG-4	Phenol/Phenol	-
HB101 (TOM$_{31c}$)	MFL-1	Toluene/Toluene	-

Table 6. TRANSCONJUGATIONAL STRATEGIES TESTED
(continued)

Donor	Recipient	Selection Donor/Recipient	Growth[1]
HB101 (TOM$_{31c}$)	MFL-2	Toluene/Toluene	-
HB101 (TOM$_{31c}$)	WFBL-2	Toluene/Toluene	-
HB101 (TOM$_{31c}$)	WFBL-3	Toluene/Toluene	-
PR1 (TOM$_{31c}$)	WFBI-1	42C/Phenol	-
PR1 (TOM$_{31c}$)	WFBI-2	42C/Phenol	-
PR1 (TOM$_{31c}$)	MFG-1	Anaerobic/Phenol	-
PR1 (TOM$_{31c}$)	MFG-2	Anaerobic/Phenol	-
PR1 (TOM$_{31c}$)	MFG-3	42C/Phenol	-
PR1 (TOM$_{31c}$)	MFG-4	42C/Phenol	-
PR1 (TOM$_{31c}$)	MFL-1	Anaerobic/Then toluene	-
PR1 (TOM$_{31c}$)	MFL-2	Anaerobic/Then toluene	-
PR1 (TOM$_{31c}$)	WFBL-2	Anaerobic/Then toluene	-
PR1 (TOM$_{31c}$)	WFBL-3	Anaerobic/Then toluene	-
249-2 (TOM$_{31c}$)	BR-14	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	BR-15	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	BR-17	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	WS-19	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	WS-21	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	WS-22	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	WS-23	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	WS-25	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	MF-A	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	MF-B	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	MFE-2	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	NFL-1	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	NFG-1	No lysine/Toluene	-

Table 6. TRANSCONJUGATIONAL STRATEGIES TESTED
(continued)

		Selection	
Donor	Recipient	Donor/Recipient	Growth[1]
249-2 (TOM$_{31c}$)	NFE-2	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	NFE-1	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	WF-GATT-A	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	WFA-4	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	NFL-3	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	BR-27	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	NFG-2	No lysine/Toluene	-
249-2 (TOM$_{31c}$)	MFI-1	Km/Nal on R2A	-
249-2 (TOM$_{31c}$)	MKPNL9cr	Km/Cm on R2A	-
249-2 (TOM$_{31c}$)	WSL-1	Km/Cm on R2A	-
249-2 (TOM$_{31c}$)	MFI-1	Km/Cm on R2A	-
249-2 (TOM$_{31c}$)	UWI-1	Km/PIP on R2A	-
249-2 (TOM$_{31c}$)	EPI-1	phenol/PIP	-
249-2 (TOM$_{31c}$)	MFG-3	Km/Ap on R2A	+
249-2 (TOM$_{31c}$)	WFBL-4	Phenol/PIP	-
249-2 (TOM$_{31c}$)	WF-GATT-B	Phenol/PIP	-
249-2 (TOM$_{31c}$)	**NFL-2**	**Phenol**	**+**
249-2 (TOM$_{31c}$)	**UWL-1**	**phenol**	**+**
HB101 (TOM$_{31c}$)	**A2-1**	**Km/Ap on R2A**	**+**
HB101 (TOM$_{31c}$)	MFE-2	Km/Ap on R2A	-
HB101 (TOM$_{31c}$)	MFG-1	Km/Ap on R2A	-
HB101 (TOM$_{31c}$)	MFG-3	Km/Ap on R2A	-
HB101 (TOM$_{31c}$)	MFG-4	Km/Ap on R2A	+
HB101 (TOM$_{31c}$)	MKPNL9cr	Km/Ap on R2A	-
HB101 (TOM$_{31c}$)	**NFG-2**	**Km/Ap on R2A**	**+**
HB101 (TOM$_{31c}$)	WSL-1	Km/Ap on R2A	-

Table 6. TRANSCONJUGATIONAL STRATEGIES TESTED
(continued)

Donor	Recipient	Selection Donor/Recipient	Growth[1]
HB101 (TOM$_{31c}$)	MFI-1	Km/Ap on R2A	-
HB101 (TOM$_{31c}$)	MFI-1	Km/Nal on R2A	-
HB101 (TOM$_{31c}$)	MKPNL9cr	Km/Cm on R2A	-
HB101 (TOM$_{31c}$)	WSL-1	Km/Cm on R2A	-
HB101 (TOM$_{31c}$)	MFI-1	Km/Cm on R2A	-
HB101 (TOM$_{31c}$)	**EPI-1**	**phenol**	**+**
HB101 (TOM$_{31c}$)	NFL-2	phenol	-
HB101 (TOM$_{31c}$)	UWL-1	phenol	-
HB101 (TOM$_{31c}$)	WFBL-4	phenol	-
HB101 (TOM$_{31c}$)	WF-GATT-B	phenol	-
PR1 (TOM$_{31c}$)	MFI-1	Km/Nal on R2A	-
PR1 (TOM$_{31c}$)	MKPNL9cr	Km/Cm on R2A	-
PR1 (TOM$_{31c}$)	WSL-1	Km/Cm on R2A	-
PR1 (TOM$_{31c}$)	**MFI-1**	**Km/Cm on R2A**	-
PR1 (TOM$_{31c}$)	UWI-1	Km/PIP on R2A	-
PR1 (TOM$_{31c}$)	EPI-1	Phenol/PIP	-
PR1 (TOM$_{31c}$)	WFBL-4	Phenol/PIP	-
PR1 (TOM$_{31c}$)	WF-GATT-B	Phenol/PIP	-
WS-23 (pTom 31c)	MFI-1	Km/Nal on R2A	-
MF-4(TOM$_{31c}$)	MFI-1	Km/Nal on R2A	-
MFG-2 (TOM$_{31c}$)	MFI-1	Km/Nal on R2A	-
MFG-2 (TOM$_{31c}$)	MKPNL9cr	Km/Cm on R2A	-
MFG-2 (TOM$_{31c}$)	WSL-1	Km/Cm on R2A	+
MFG-2 (TOM$_{31c}$)	MFI-1	Km/Cm on R2A	-
MFG-2 (TOM$_{31c}$)	UWI-1	Km/PIP on R2A	-
MFG-2 (TOM$_{31c}$)	EPI-1	Phenol/PIP	-
MFG-2 (TOM$_{31c}$)	WFBL-4	Phenol/PIP	-

Table 6. TRANSCONJUGATIONAL STRATEGIES TESTED
(concluded)

Donor	Recipient	Selection Donor/Recipient	Growth[1]
MFG-2 (TOM$_{31c}$)	WF-GATT-BPhenol/PIP		-
BR-5 (TOM$_{31c}$)	A2-1	Km/Ap on R2A	-
BR-5 (TOM$_{31c}$)	MFE-2	Km/Ap on **R2A**	-
BR-5 (TOM$_{31c}$)	MFG-1	Km/Ap on R2A	-
BR-5 (TOM$_{31c}$)	MFG-3	Km/Ap on R2A	-
BR-5 (TOM$_{31c}$)	MFG-4	Km/Ap on R2A	-
BR-5 (TOM$_{31c}$)	MKPNL9cr	Km/Ap on R2A	-
BR-5 (TOM$_{31c}$)	NFG-2	Km/Ap on R2A	-
BR-5 (TOM$_{31c}$)	WSL-1	Km/Ap on R2A	-
BR-5 (TOM$_{31c}$)	MFI-1	Km/Ap on R2A	-
BR-5 (TOM$_{31c}$)	MFI-1	Km/Nal on R2A	-
BR-5 (TOM$_{31c}$)	MKPNL9cr	Km/Cm on R2A	-
BR-5 (TOM$_{31c}$)	WSL-1	Km/Cm on R2A	-
BR-5 (TOM$_{31c}$)	MFI-1	Km/Cm on R2A	-
BR-5 (TOM$_{31c}$)	UWI-1	Km/PIP on R2A	-
BR-5 (TOM$_{31c}$)	EPI-1	phenol	-
BR-5 (TOM$_{31c}$)	NFL-2	phenol	-
BR-5 (TOM$_{31c}$)	UWL-1	phenol	-
BR-5 (TOM$_{31c}$)	WFBL-4	phenol	-
BR-5 (TOM$_{31c}$)	WF-GATT-Bphenol		-

[1]**Bold type** indicates that the presumptive TOM$_{31c}$ transconjugant was confirmed later by more specific tests (TFMP (trifluoromethyl phenol) and TCE oxidation tests, plasmid isolation and DNA hybridization). All other + growth presumptives were later shown not to be the expected strain or did not contain TOM$_{31c}$.

4.   Biolistic Transformation

TOM$_{31c}$ DNA was purified from HB101(TOM$_{31c}$) using a modified method of
Brimboim for large-scale plasmid extraction and CsCl$_2$ purification. The microbial strains
employed were selected on the basis of their availability, kanamycin sensitivity, and inability to
grow with p..no! as th.. ..ole source of carbon and energy. These strains were grown overnight
in R2A media at 30°C, with shaking at 150 rpm. Using the protocol presented by Smith, et
al., 1992 (13) as a guide, the plasmid DNA was coated onto M5 tungsten particles specially
prepared and handled so as to prevent agglomeration. These particles were then "shot" onto
"pagar" spread with 3 X 10^9 cells of each of the selected strains. Cell number was determined
using optical density at 600 nm measurements of suspensions of washed cells. The pagar was
transferred directly onto the selective media and incubated overnight. Both rupture pressure and
flight distance was varied in order to obtain putative transconjugants. Aliquots of the cell
suspensions were spread onto R2A plates as positive controls for growth and un-inoculated R2A
plates were bombarded without tungsten as a negative control.

5.   Electroporation

The electroporation protocol of the manufacturer (BRL Cell Porator and Voltage
Booster) was followed. In addition, attempts were made to also transform selected gram (+)
strains via electroporation (2) where various concentrations of glycine were used to weaken the
cell walls before electroporation. Because of time constraints, only a concentration of 2.5%
glycine (a moderate amount of glycine) was utilized in these experiments.
In each attempt following electroporation the bacteria were incubated at 30°C for
1.5 hrs. 150 μL were plated to selective media. Afterwards, the cells were centrifuged (2 min.
at 14 K) and a 150 μL aliquot of the supernatant was plated to the same medium in case a
particularly low efficiency of transformation had resulted.

6.   TFMP Oxidation Assay for Tom Activity

One of the products in this section is the development of this assay. The A$_{600}$ of
the culture is recorded. 1 mL is pelleted in a microfuge (15,000 rpm for 30 seconds) and

resuspended in 1mL of 10mM Tris-Cl pH 8.5 1.0mM triflouromethyl phenol (TFMP) (also: m-hydroxy benzo triflouride). These cells are then incubated in a 25 mL Erlenmeyer flask at 30°C for 20 minutes open to the air. The process to this point can be used as a qualitative assay for yellow color development (i.e. triflouroheptadienoic acid (TFHA) production [molar extinction coefficient at 385 nm = 26,900 AU.L/mole]). In order to quantitate the enzyme activity the cells are again pelleted in a 1.5 mL microfuge tube, 15,000 rpm/ 30 seconds. The supernatant is transferred to a clean tube and placed on ice until ready to read. Care must be taken to avoid any cellular material. The A_{386} and A_{600} of each cleared supernatant sample is recorded. Since the yellow TFHA product absorbs strongly at 386 but not at 600 nm the A_{386} absorption caused by light diffraction of whole cells can be subtracted by measuring the ratio of A_{386} /A_{600} (using a low concentration of cells: where the A_{600} is < 0.1). This allows conversion of the A_{386} measured to a corrected A_{386}.

$$A386 \text{ corrected} = A386_{\text{measured}} - A386_{\text{due to diffractive loss}}$$
$$= A386 \text{ measured} - [(A600 \text{ sample}) \times (C)]$$

Where C is the A_{386} /A_{600} ratio .

Calculations:

mg Protein/mL (for G4 strains) = A600 x 0.290

micromolar TFHA = A386 corrected/ 0.0269

nmoles TFHA = μM x 0.001L x 1000 nmol /μmol

nmoles TFHA/min = nmol TFHA/20 min

Specific activity: nmoles TFHA/min/mg protein

7.   Molecular Techniques.

E. coli and *B. cepacia* plasmids were isolated by the Birnboim and Doly alkaline lysis technique (1). Southern Blot, nick translation, and autoradiography were performed according to Maniatis (7).

The sequence of the cloned *tom*A gene region used as a [32]-P-labeled, ssDNA probe is presented in Figure 13.

1
GGATCCCGAGCATGAACTGTTCGACACGTCGCTGCGGTACCGCGATTCAAATGAAGGACTGGTATGCGCTGAAAC...CCCGCGTC
AGTTCTACTACGCGTCGTGGACGATGACCCGAGCGCGGCAAGACGCGCAGCAAGACGCGATGGAATCCAACTTCGAGTTTGTCGAGTCG
CGCGGCATGATCGATCTCGTTCCGATGAGGTTCGACAACGGGCGCTTCCGTTCTCGTGCCTTTGCCTTGCCGTCACGCGGCCTG
GGGCGCGAACATGAACAACTCCCAGATCTGTGCCCTAGGTTATGGCACGACCTTCACTGCGCGCGGCTATGTTCCACGCAA
TGGACAAATCTGGGTGTGTAGCGCAGTATCTCACACGACTGGCGCTGGTAATGTCTGGAAAACACTCTGGTGCTGCAAGATCCGGTGGA
CAAGCCTGGATGACGAGTCCCGATTGGCAACCGTTGCCGTCGTCGTTATGTGGAAAATCCCATGATCTACGGCGCTTTCGTCGACGATTACATCG
ACTGTTCATCGCCCAAAATCTGGCGCTCGACGGTCAATGCTAACCACTTTCATGCCCGAGTGGCATGACGAATCCAGTCGCTGGGTCGAT
CACTCAACGGTGGTAGCGCAGTGGCAATGCTAACCACTTTCATGCCCGAGTGGCATGACGAATCCAGTCGCTGGGTCGAT
GCGGTAGTAAAGACCATGGCGACGGAATCGGAGGATAACAAAGCGCTGCTCATTCACTGGTTGCGTACCTGGGAAGATCA
GGCGGCGTCAGCGTTGTTGCCTGTCGCTGAAATGGCTTTGCGCTGTAACCCCTAATTTATATTCCTGACCGTCATAGAGGAATGTTC
AACTTCGTGCCCGCGTTGCGAAGGCCGGGATTGTTCTGTAACGAGGAGTCTCGACCGGTGGTGGAGGCGATTCTCGCTGATAACCCTAA
ATGTCTAACGTATTTATCGCGTTTCAGGCTAAGGCATGGTGAAGATCGATGCGCCCGGGCATCTCACAATAAACCGTCAAAGCATCGAAG
GGCGGTCGCCACAGAATCGCCTGGCATGGTGAAGATCGATGCGCCCGGGCATCTCACAATAAACCGTCAAAGCATCGAAG
ACCGGATCGGCATGAAGTTCGATCTCCAGCAAATCCACATCAACCTGTCCGGATATATCGACGAGGATGAC
GAACAGTTCACGCTGAGCTGGAAACACTGAACACGGCAAAGAGGAAATTGAAATGGACACTTCTGTGCAGAAGAGAAAC
TCGGTTTAAAGGATCGCTACGCAGGCATCAAGATCCACGATTGGGATAAATGGGAAGACCCCTTCCGCCTGACCATGGACGCCCTA
CCGTACGACAAGTACGAAGGCATCAAGATCCACGATTGGGATAAATGGGAAGACCCCTTCCGCCTGACCATGGACGCCCTA
CTGGAAATATCAGGGCGGAGAAGGAAAAA
1388

Figure 13. DNA sequence of the *tomA* gene

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C.   RESULTS AND DISCUSSION

1.   Transfer of Constitutive Plasmids to Alternative Bacterial Strains

a.   Potentially Selectable Field Application Vector Hosts

Transfer of the self conjugative, Tom constitutive plasmids (TOM_{31c} and TOM_{301c}) to the FAVs identified in section I was attempted. Because of the large size of these plasmids (112 and c.a. 107 kb respectively) their introduction to other bacteria was approached with three techniques: conjugation, electroporation and biolistic transformation.

Biolistic transformation is by far the most unusual of techniques employed. These involved the attempted introduction of $CsCl_2$ purified plasmid TOM_{31c} into selected microbial FAV candidate strains by accelerating plasmid coated tungsten particles into prepared cells at high velocities in a stream of compressed He.

(1)   Conjugation with TOM_{31c}

Plate surface and membrane matings were attempted with either *E. coli* or *B. cepacia* carrying TOM_{31c}. These donor strains were constructed because they are both auxotrophs (proline and lysine, respectively) and as such are far more readily counterselectable than $PR1_{31}$.

The results of these conjugational mating attempts are shown in Table 6.

(2)   Conjugation with TOM_{301c}

Using the antibiotic and carbon source utilization profiles of each of the available FAVs which were Gram -, Phe⁻, and Km*, appropriate selections were chosen to be used in the a conjugal filter mating attempt. Potential donors (in this case $PR1_{31}(TOM_{301c})$) and recipient broth cultures were mixed, filtered and allowed to mate on a non-selective medium. The plasmid TOM_{301c} does not carry a Tn*5* insertion; therefore it does not confer Km^r to its host. Thus, other selections were considered. In addition, potential crosses were determined for selected Gram + FAVs (WFBI-1 and WFBI-2 only) as recipients for the plasmid

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TOM$_{301c}$ using double antibiotic selections. Table 7 indicates the possible selections initiated for each of the donor and recipient pairs examined.

All matings were attempted twice without success. It is possible that not all of the TMP or RA went into solution during preparation of the stock solution used to make the selection plates, the undissolved portion being removed by the filter during sterilization of said stock solution. This might have influenced the actual final concentration of TMP or RA in the selection plates. Because of time constraints, other possible selections with each donor/recipient pair were not attempted, but may be possible and should definitely be considered in any future plans.

Table 7. POSSIBLE CONJUGAL OR FILTER MATING SCHEMES

SELECTION	DONOR	RECIPIENTS
2mM Phe 5 μg/mL TMP	PR131 (pTOM$_{301c}$)	A2-1 EPI-1 MF-4 MFG-2 MKPNL9cr NFG-2 WFBL-4 WFGATTB MFI-1
2mM Phe 50 μg/mL Kn	PR131 (pTOM$_{301C}$)	EPI-1 WFBL-4 WFGATTB
2mM Phe 50 μg/mL Cm	PR131 (pTOM$_{301C}$)	WSL-1
0.5 mM o-Cr 5 μg/mL RA	PR131 (pTOM$_{301C}$)	MFE-2
0.5 mM o-Cr 5 μg/mL TMP	PR131 (pTOM$_{301C}$)	WSE-2
R2A 100 μg/mL Amp 5 μg/mL TMP	PR131 (pTOM$_{301c}$)	UWL-1 WFBI-1 g(+) WFBI-2 g(+)

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(3)   Electroporation with TOM$_{31c}$

Attempts were made to introduce the plasmid TOM$_{31c}$ into selected microbial strains by electroporation. This method involves exposing bacteria in a carefully adjusted electrolyte solution to a short electric potential in the presence of the desired plasmid.

Attempts were made to electroporate those Gram -, Phe$^-$ Km^s FAV strains which so far have proven difficult to transform with previously attempted alternate protocols. In addition several Gram + strains were selected and tested. Voltages were varied in these attempts; both a low voltage (312.5v) and a high voltage (375v) were tested with all strains. Also, the pulse lengths tests were 6 ms and 12 ms (changed by varying the resistance settings on the voltage booster). DNA concentrations tested were 1μg and 10 μg/20μl of electroporated culture. Gram negative strains tested were: JM109, MFE-2, MFG-1, MKPNLcr, WSL-1. Gram positive strains tested were: WFBI-1 and WFBI-2.

Only *E. coli* controls receiving relatively small plasmids (pGEM4Z or pMS64) were successfully electroporated. In no case was TOM$_{31c}$ transfer to a recipient by this technique detected.

(4)   Biolistic Transformation with TOM$_{31c}$

Biolistic transformation of several strains was attempted with TOM$_{31c}$ under several conditions where the physical factors were varied without success (including flight distance, rupture pressure, etc.). We did achieve the biolistic transformation of the *E. coli* positive control with the small cloning vector pGEM4Z.

Colonial morphologies of several putative transconjugants proved to be contaminants following cultural, and PCR analyses (which amplified TOM$_{31c}$ plasmid DNA controls using primers of the upstream constitutive element). In addition, TFMP analyses of each putative transconjugant was negative and antibiotic resistance profile comparisons to parent strains showed significant differences.

Why this technique should work for the 2.9 kb cloning vector, but not for the 112 kb TOM$_{31c}$ is unclear in the face of such an apparent large scale traumatic mechanism. While this is certainly a much larger plasmid (39 times) it is still far smaller than the W particles used to "punch" holes in the recipient bacterium. One possible reason for this

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difference would be a difference in how the DNA binds the metal surface (ostensibly as a torroid due to pretreatment with spermidine).

b.   Dominant Indigenous Bacterial Hosts

Because it may be possible to merely reintroduce a dominant bacterium to a contaminated aquifer and have it maintain itself as it was doing before our intervention it may be possible to introduce TOM_{31c} to this population and achieve remediation without the need for auxiliary treatments. To test this, TOM_{31c} was first transferred to seven bacterial strains, not isolated as FAVs, but rather as representing dominant organisms grown on R2A from Borden aquifer material: $BSE3-1(TOM_{31c})$, $BSE22-1(TOM_{31c})$, $BSE12-1(TOM_{31c})$, $BSE14-1(TOM_{31c})$, $BSE11-1(TOM_{31c})$, $BSE2-3(TOM_{31c})$, and $BSE1-2(TOM_{31c})$ Transfer was via conjugational matings with $PR1_{31c}$, and selection was based on strains selected for Km^s, and subsequent conversion to Km^r following conjugation with $PR1_{31}$. Assays to assess biodegradative potential of these transconjugants were performed to determine the new transconjugants ability to degrade TCE.

The FAV and dominant bacterial transconjugates were labeled according to their source and (where appropriate) method of isolation. Their attributes are summarized in Table 8.

2.   TCE Degradation and TFMP Oxidation By Transconjugants

a.   TCE pentane extraction assays.

(1)   FAVs

BR-5, BR-5 (TOM_{31c}), MFG-2, MFG-2 (TOM_{31c}), and 249-2 (TOM_{31c}) (the donor) were grown overnight in R2A broth, and tested for TCE degradation potential via the pentane extraction assay (at an initial concentration of approximately 500 ug/L). A negative control containing only R2A and TCE was run as in addition to the bacteria. Transconjugates MFG-2 (TOM_{31c}), NFG-2 (TOM_{31c}) and MFI-1 (TOM_{31c}) successfully degraded TCE (Figures 14 and 15).
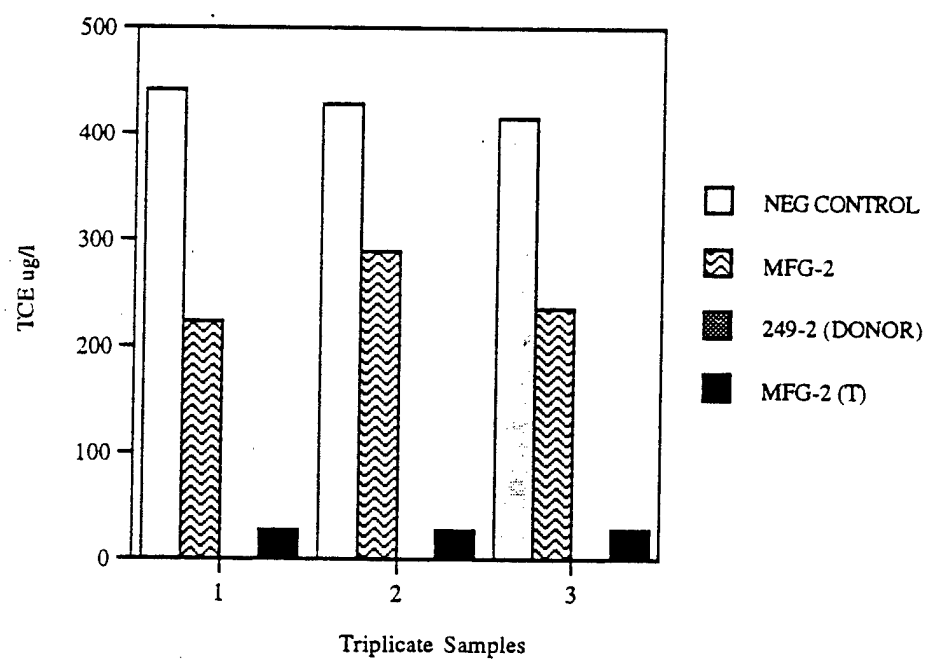
Table 8. TRANSCONJUGANTS

FAVs

	Carbon Enrichment	TOM_{31c} Donor	TCE Degradation	Km^r
MFG-2	20 mM glucose	249-2 (TOM_{31c})	+	+
NFG-2	20 mM glucose	HB101 (TOM_{31c})	+	+
MFI-1	0.1% IGEPAL	MFG-2 (TOM_{31c})	+	+
EPI-1	0.1% IGEPAL	HB101 (TOM_{31c})	-	+
NFL-2	20 mM lactate	249-2 (TOM_{31c})	-	+
UWL-1	20 mM lactate	249-2 (TOM_{31c})	-	+

Predominant Strains

MF-4	none	249-2 (TOM_{31c})	+	+
WS-23	none	249-2 (TOM_{31c})	+	+
BR-5	none	249-2 (TOM_{31c})	-	-
BSE3-1	none	249-2 (TOM_{31c})	+	+
BSE22-1	none	249-2 (TOM_{31c})	+	+
BSE12-1	none	249-2 (TOM_{31c})	+	+
BSE14-1	none	249-2 (TOM_{31c})	+	+
BSE11-1	none	249-2 (TOM_{31c})	+	+
BSE2-3	none	249-2 (TOM_{31c})	+	+
BSE1-1	none	249-2 (TOM_{31c})	+	+
BSE24-1	none	249-2 (TOM_{31c})	-	+
A2-1	none(nitrogen fixer)	HB101 (TOM_{31c})	-	+

(2)   Dominant Indigenous Strains

MF-4, WS-23, BSE1, BSE2, BSE3, BSE22, BSE12, BSE14, BSE11, BSE24 and their TOM_{31c} transconjugants were examined by this technique and all but one constitutively degraded TCE (Figures 16-21).

FIGURE 14. TCE Degradation Assay for MFG-2, MFG-2 (TOM$_{31c}$)

MFI-1 (TOM$_{31c}$)

NFG-2 (TOM$_{31c}$)
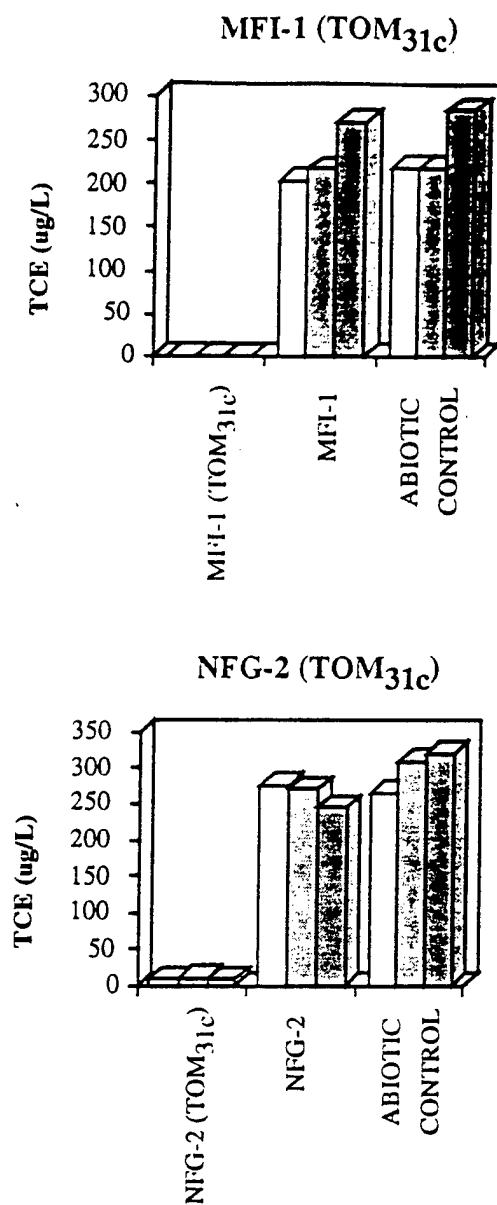
Figure 15. TCE Degradation by the Pentane Extraction Assay

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WS-23 and WS-23 (TOM$_{31c}$)

Legend:
- Uninoculated Control
- BR-23
- 249-2 (TOM$_{31c}$)
- BR-23 (TOM$_{31c}$)

MF-4 and MF-4 (TOM$_{31c}$)

Legend:
- NEG CONTROL
- MFE-4
- 249-2 (TOM$_{31c}$)
- MFE-4 (TOM$_{31c}$)
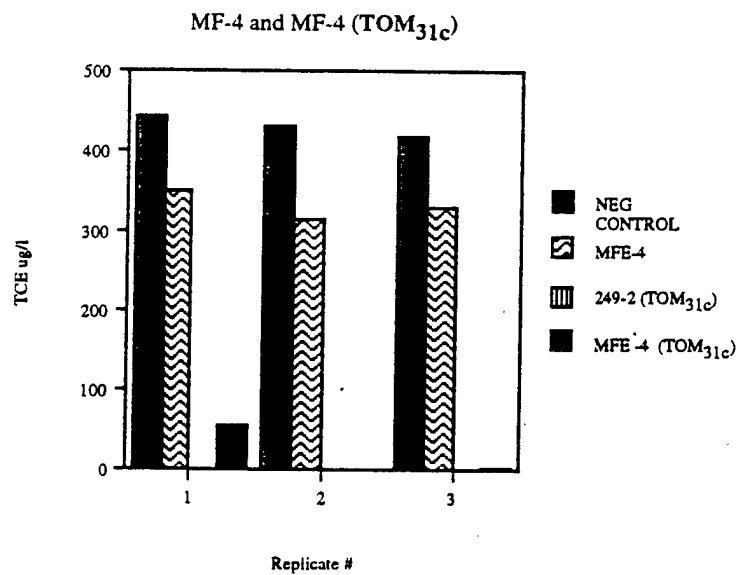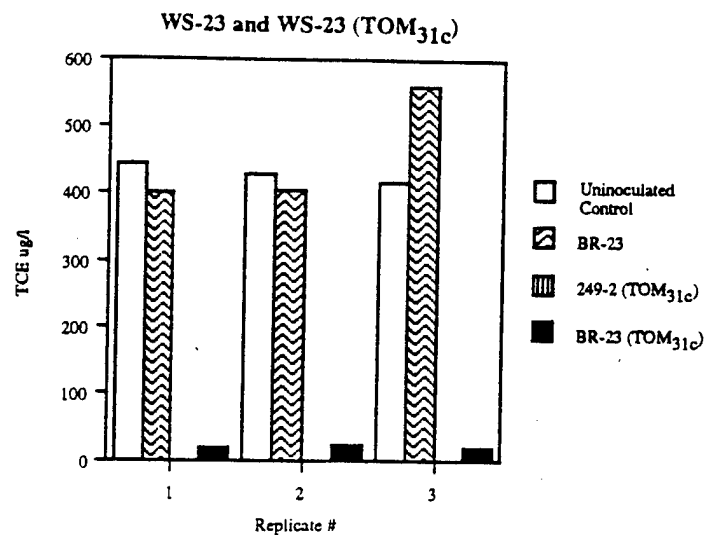
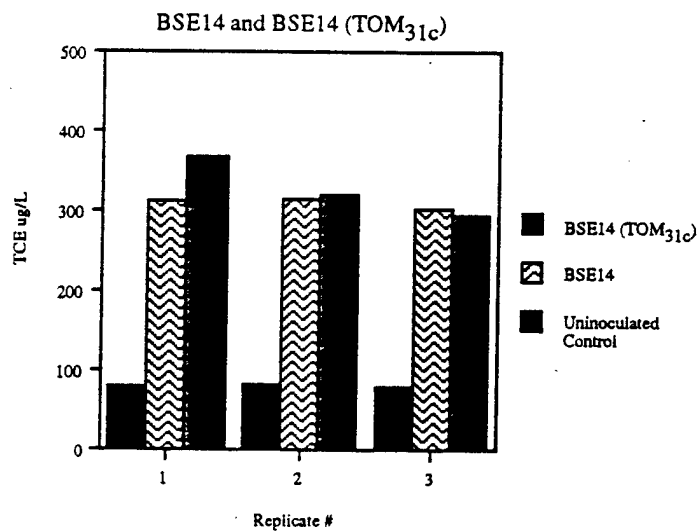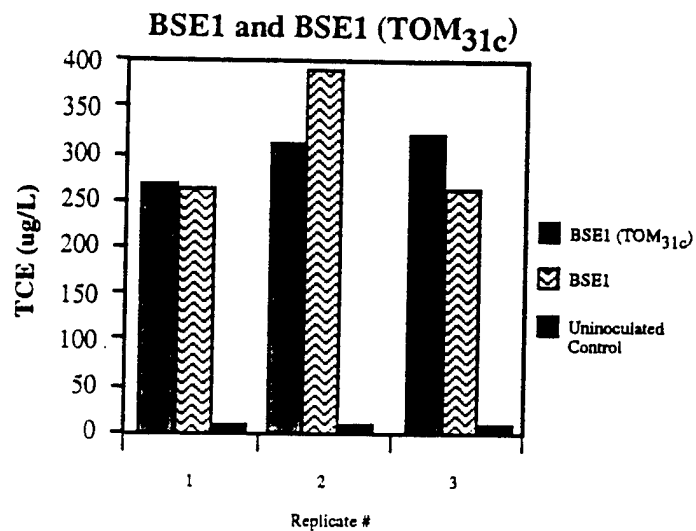**Figure 16. TCE Remaining After Exposure to MF-4 and WS-23 Transconjugants**
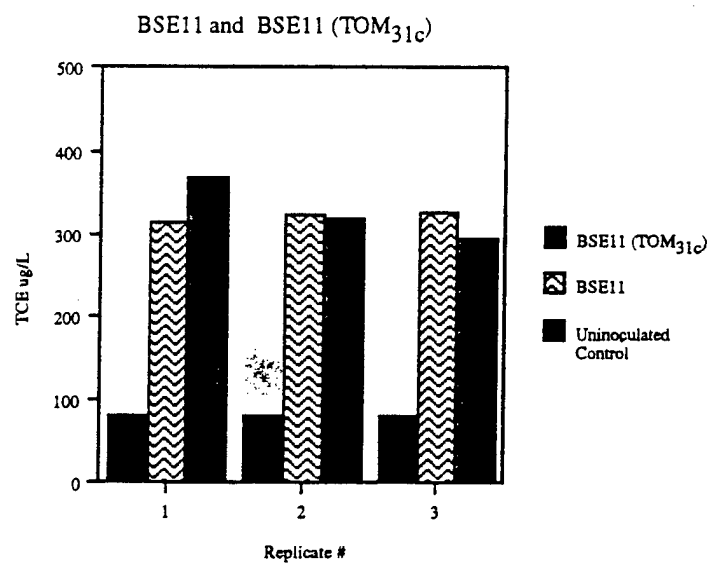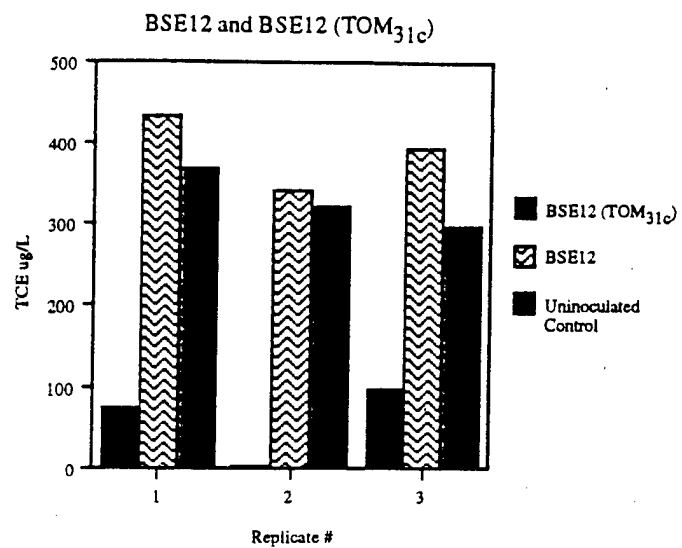
Figure 17. TCE Remaining After Exposure to BSE-11 and BSE-12 Transconjugants

Figure 18. TCE Remaining After Exposure to BSE-1 and BSE-14 Transconjugants

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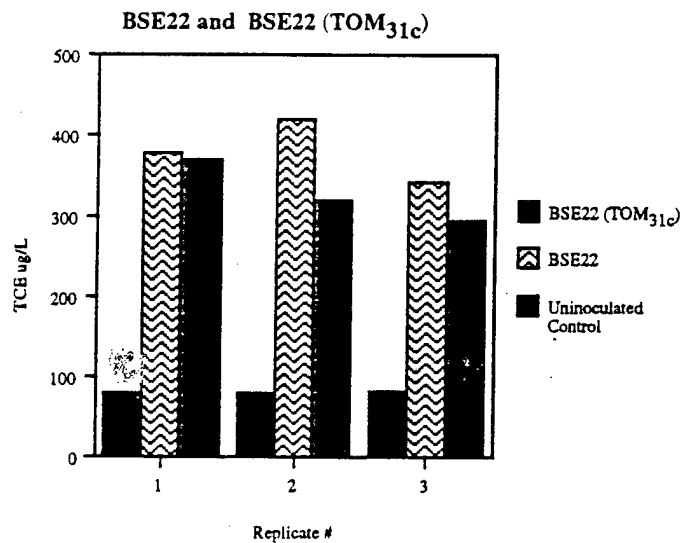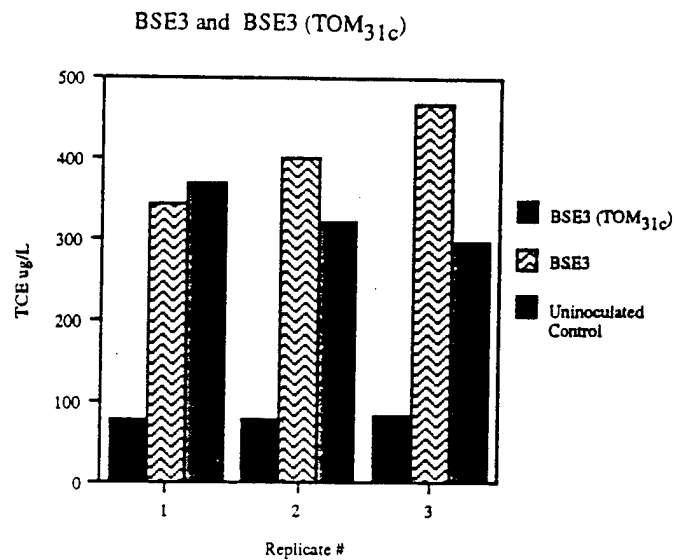
Figure 19. TCE Remaining After Exposure to BSE-3 and BSE-22 Transconjugants
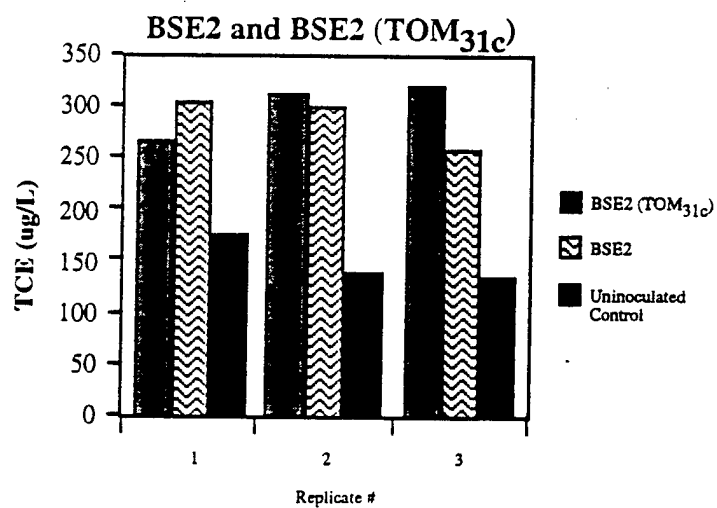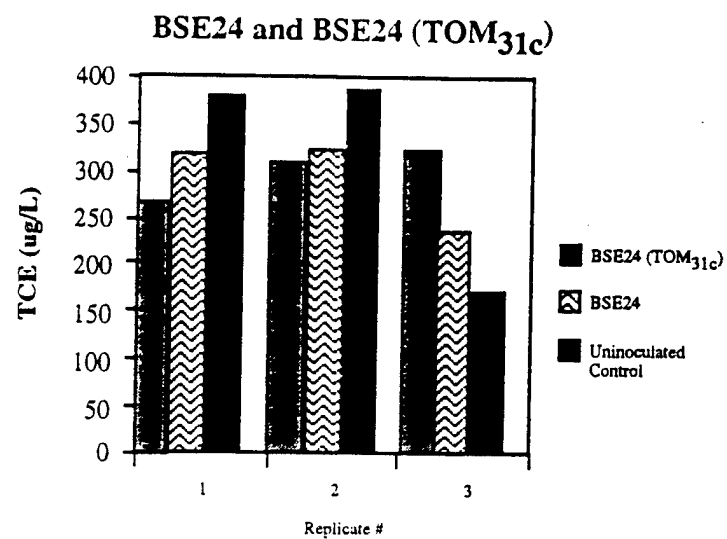
Figure 20. TCE Remaining After Exposure to BSE-2 and BSE-24 Transconjugants

b.　　TCE Air Headspace Assay.

The FAVs: MFI-1, MFI-1 (TOM$_{31c}$), NFG-2, NFG-2 (TOM$_{31c}$), MFI-1, MFI-1 (TOM$_{31c}$), NFG-2, NFG-2 (TOM$_{31c}$); and dominant isolates: MF-4, MF-4 (TOM$_{31c}$), WS-23, WS-23 (TOM$_{31c}$), BR-5, and BR-5 (TOM$_{31c}$), were examined for TCE degradation by air headspace to determine relative rates and extent of degradation. The results are presented in Figures 22(MF-4), 23 (WS-23), 24 (MFG-2), 25 (BR-5), 26 (MFI-1), 27 (NFG-2) and 28 (the PR1$_{31}$ and uninoculated controls).

The transconjugates WS-23 (TOM$_{31c}$), MFG-2 (TOM$_{31c}$), NFG-2 (TOM$_{31c}$), MFI-1 (TOM$_{31c}$), and MF-4 (TOM$_{31c}$) successfully at degraded TCE according to this technique. It would also appear that these transconjugants maintain and express TOM$_{31c}$ at least as well as G4 since they degrade TCE at a faster rate than PR1$_{31}$ for these preparations. It should be noted that inherent variability in activity between batches of cells prevents a clear interpretation based on one experiment as to which cell line produces the greatest Tom oxidative activity.

A summary of all transconjugants found to degrade TCE by this method is found in Table 8.

3.　　Physical Genetic Evaluation of Transconjugants

a.　　DNA:DNA hybridization.

(1)　　Southern blot.

Transconjugates were examined through molecular hybridization of Southern blots to confirm the presence of the toluene ortho-monooxygenase genes (tomA1, A2, A3, A4, A5). E. coli HB101 (TOM$_{31c}$), B. cepacia PR131 (TOM$_{31c}$), and B. cepacia 249-2 (TOM$_{31c}$) (the donors used in mating transfer experiments until now) were employed as positive hybridization controls. DNA from putative transconjugant strains MFG-2(TOM$_{31c}$), MF-4(TOM$_{31c}$), BR-5(TOM$_{31c}$), WS-23(TOM$_{31c}$), EPI-1(TOM$_{31c}$), EP-2(TOM$_{31c}$), KA-2(TOM$_{31c}$), and WFGATTB (TOM$_{31c}$) was likewise tested for homology to the tomA open reading frames in this manner.
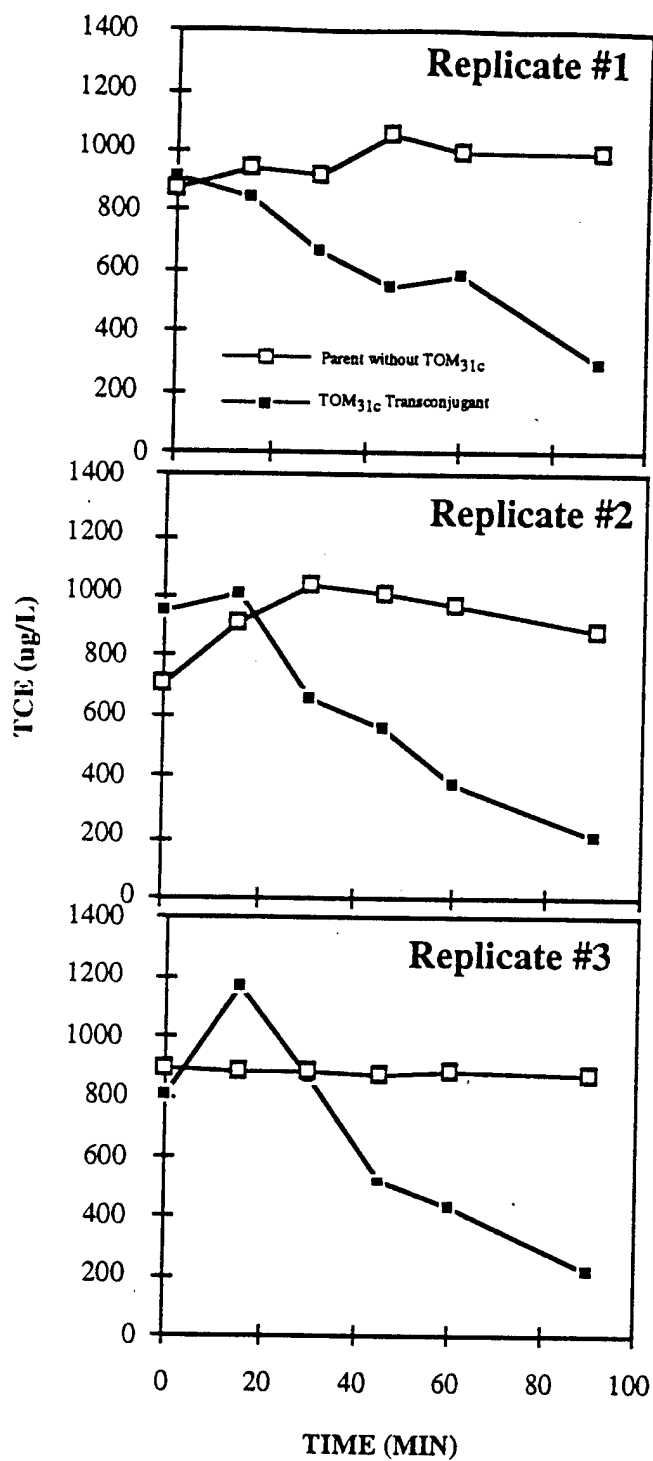
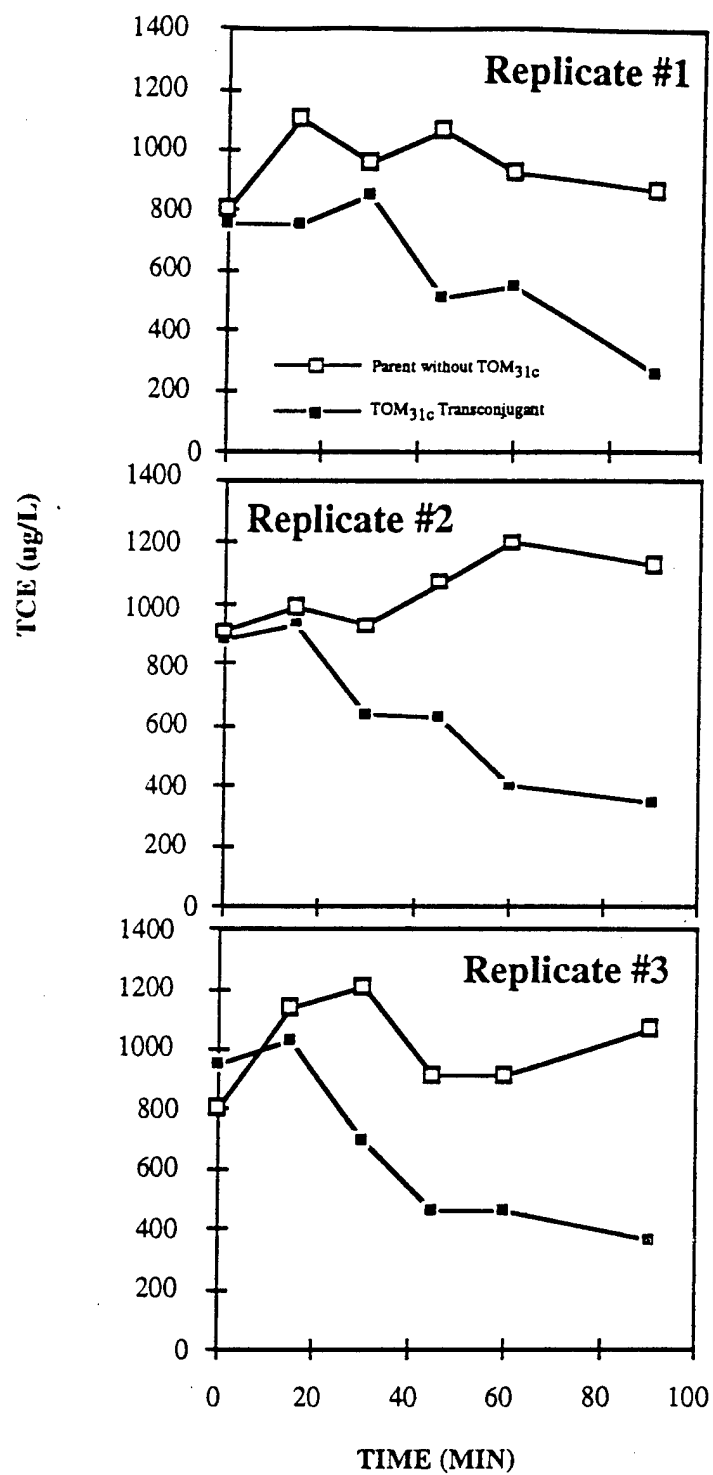Figure 21. TCE Degradation by MF-4 (TOM$_{31c}$)
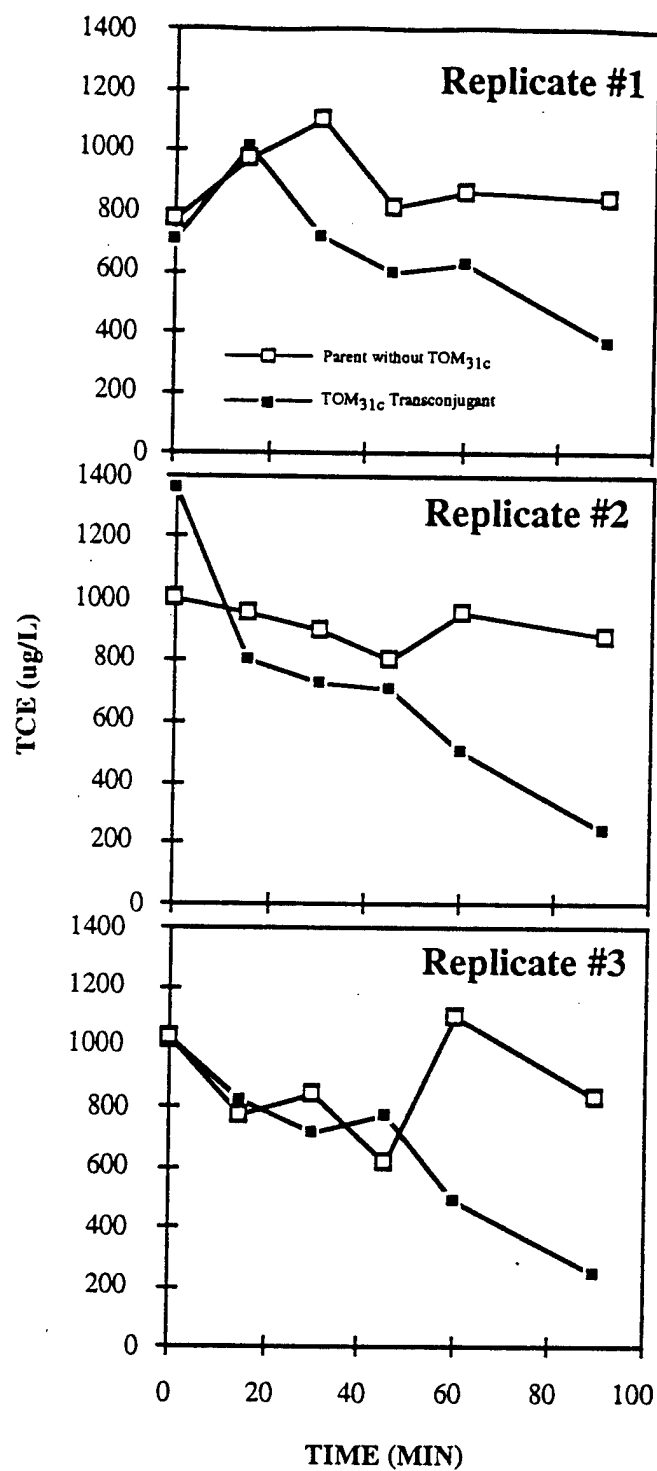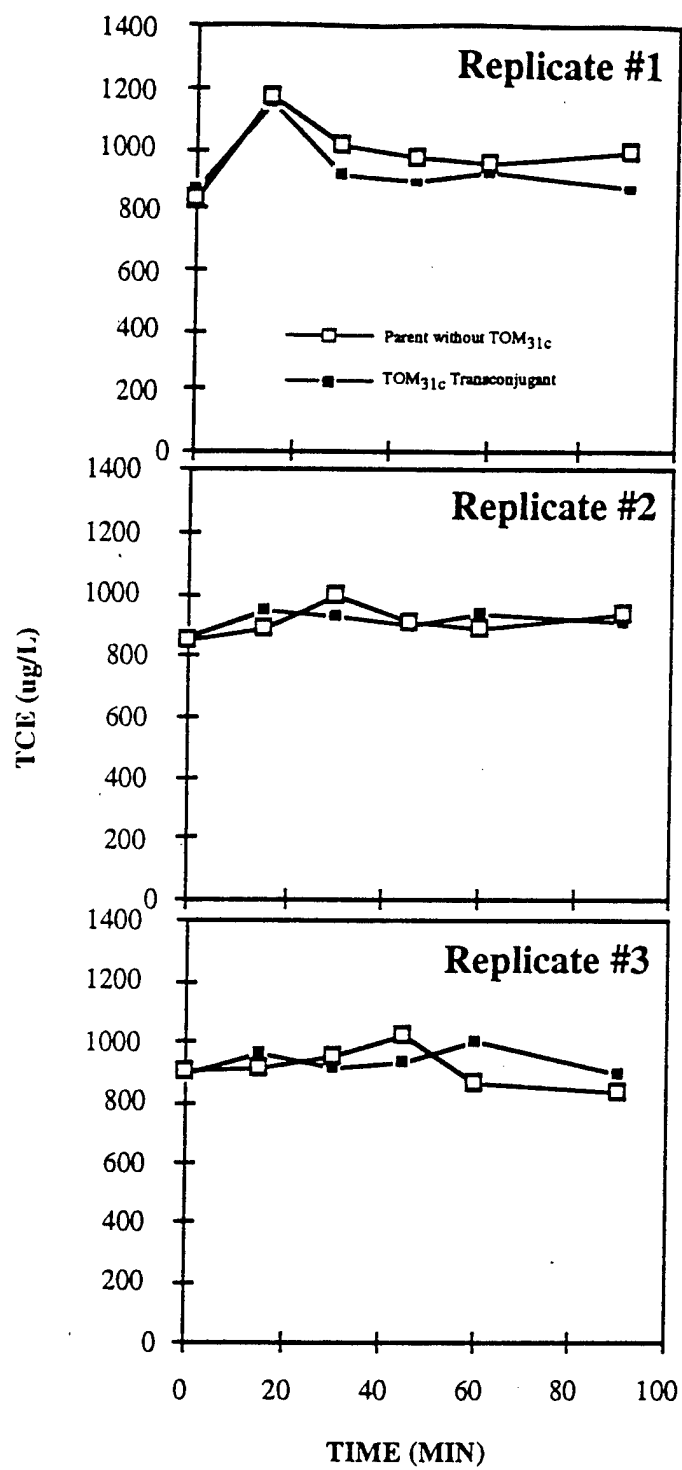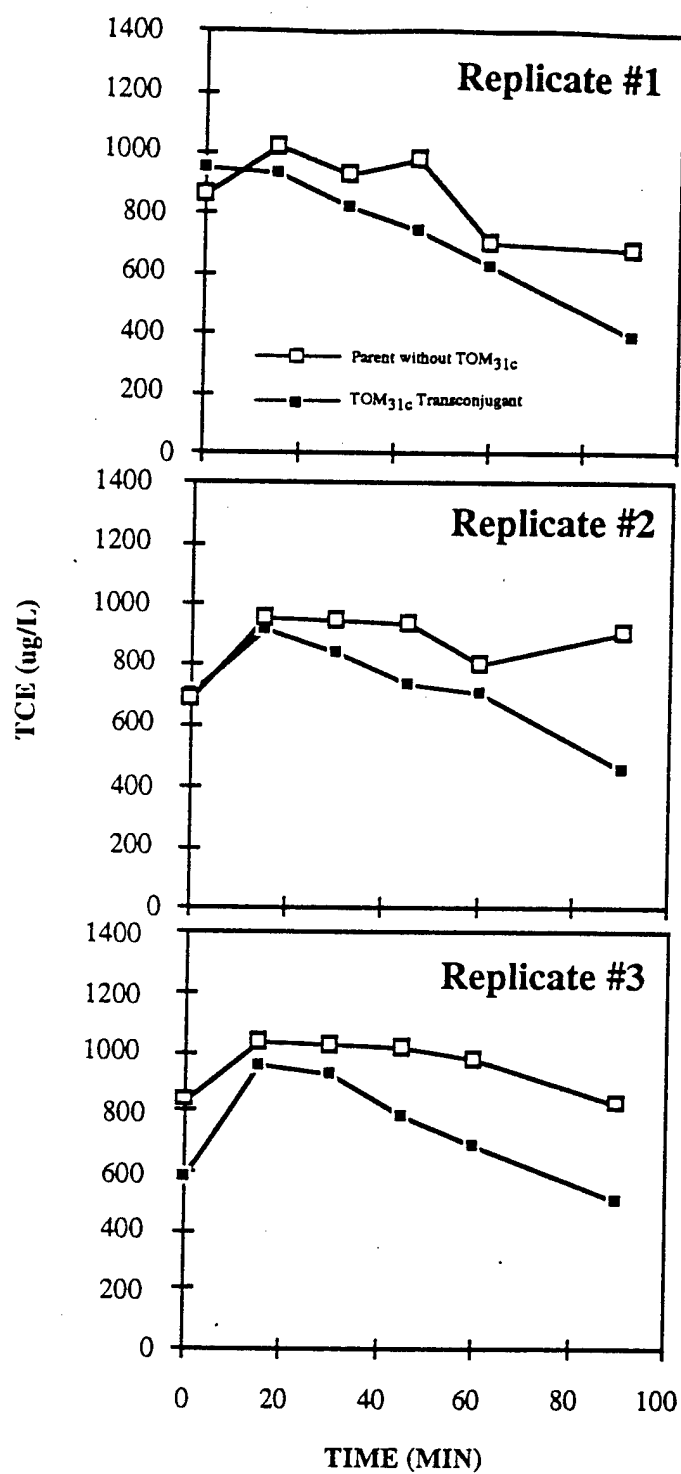
Figure 22. TCE Degradation by WS-23 (TOM$_{31c}$)

Figure 23. TCE Degradation by MFG-2 (TOM$_{31c}$)

Figure 24. TCE Degradation by BR-5 (TOM$_{31c}$)
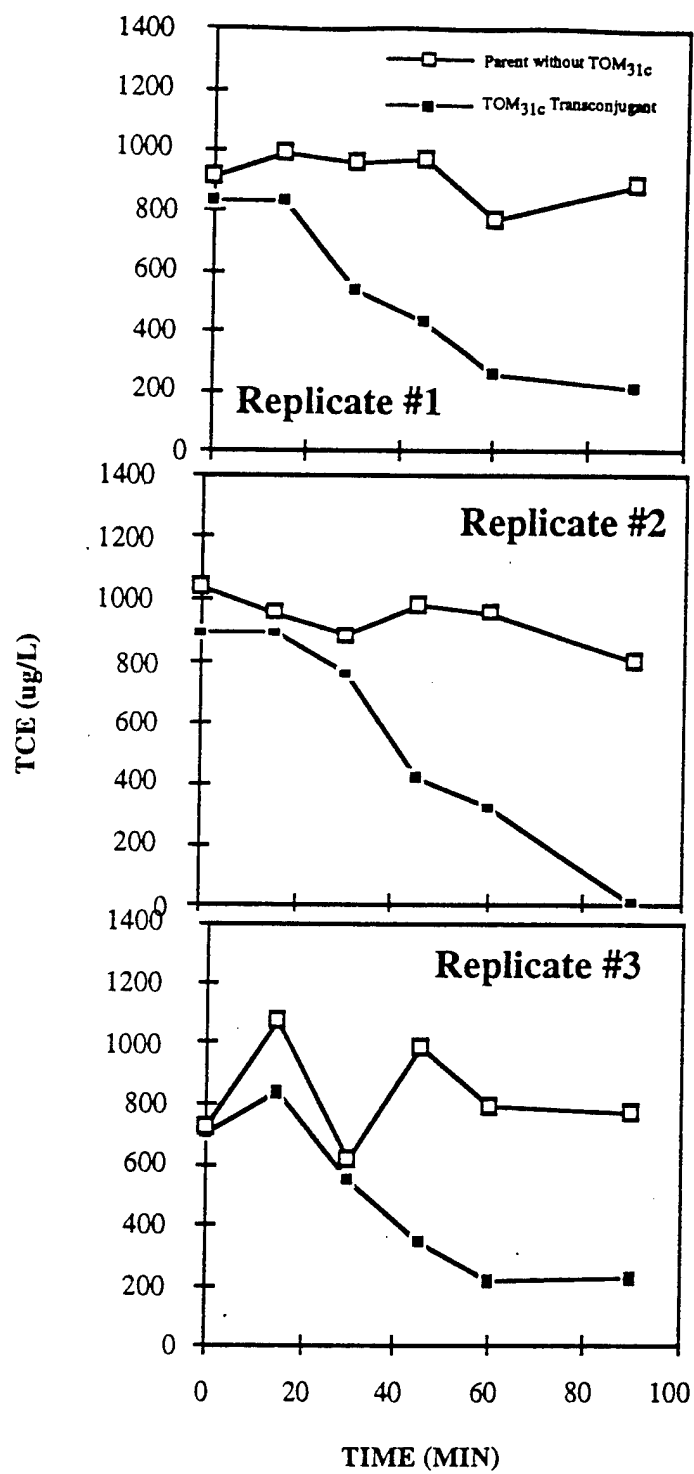
Figure 25. TCE Degradation by MFI-1 (TOM$_{31c}$)
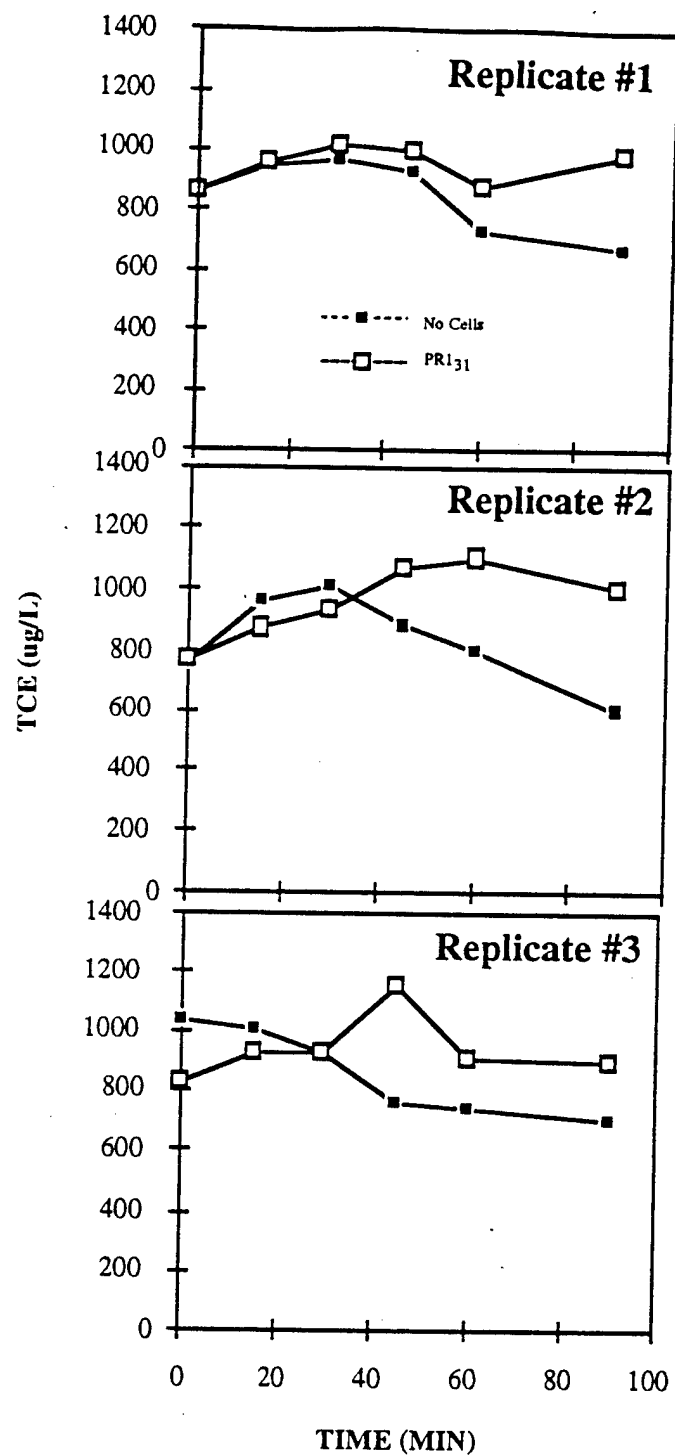
Figure 26. TCE Degradation by NFG-2 (TOM$_{31c}$)

Figure 27. TCE Degradation by PR1$_{31}$ and Uninoculated Controls.

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Hybridization results are presented in Table 9. DNA hybridization to the appropriate controls was detected, as well as to two of the six presumed transconjugants (based solely on Km^r).

(2)   Colony Hybridization.

Colony hybridizations were also performed to verify plasmid identity. These results are listed in Table 10. It should be noted that the only transconjugant to hybridize to the 11 kb *Eco*RI fragment of pMS64 containing all of *tomA* and *tomB* and IS50, without yielding an evident plasmid band was the nitrogen fixing root colonizer, A2-1.

Table 9. SOUTHERN HYBRIDIZATION EXPERIMENT

DESCRIPTION	DNA Source	Hybridization to tomA
Positive Controls	HB101 (TOM$_{31c}$)	+
	HB101 (pMS64)1	+
	G4	+
	HB101 (TOM$_{31c}$)	+
	HB101 (TOM$_{31c}$)	+
	249-2 (TOM$_{31c}$)	+
Negative	PGEM7Z	-
	PGEM4Z	-
	HB101	-
Presumed Transconjugants	MFG-4 T[2]	+
	MFE-2 T	+
	WS-23 T	+
	BR-5 T	+
	EP-2 T	-
	EPI-1 T	-
	KA-2 T	-
	WFGATTB T	-

[1]pMS64 is the source of the cloned tomABC DNA used as the probe.

[2]T indicates status as a presumptive TOM$_{31c}$ transconjugant

Table 10. COLONY HYBRIDIZATION
TO THE TOMA PROBE.

Putative

Transconjugant	Hybridization[1]
A2-1 (TOM31c)	+
UWL-1 (TOM31c)	+
NFL-2 (TOM31c)	+
EPI-1 (TOM31c)	+
MFI-1 (TOM31c)	+
NFG-2 (TOM31c)	+
MFG-3 (TOM31c)	-
WFGATTB (TOM31c)	-
Negative Controls (Recipients)	
WFGATTB	-
MFG-3	-
EPI-1	-
UWL-1	-
Positive Controls (Donors)	
PR131 (TOM31c)	+
HB101 (TOM31c)	+

[1]Hybridization to the 11 kb *Eco*RI fragment of pMS64 containing all of *tomA* and *tomB* and IS50.

b.   Plasmid Profiles.

In addition to the hybridization evidence, plasmid DNA was extracted from the presumed transconjugants (based on their Km^r phenotype) and compared to TOM$_{31c}$ extracted from PR1$_{31}$. The results of this assay demonstrated detectable plasmid DNA, co-migrating with TOM$_{31c}$ from PR1$_{31}$, HB101 and 249-2 during agarose gel electrophoresis from 5 of the 8 transconjugants tested (by the time of this experiment two

more possible transconjugants were available: MFG-4 (TOM_{31c}) and MFE-2 (TOM_{31c}).

Three putative transconjugants EPI-1 (TOM_{31c}), KA-2 (TOM_{31c}), WFGATTB (TOM_{31c}) were found not to carry TOM_{31c}. MFG-4 (TOM_{31c}), MFE-2 (TOM_{31c}), WS-23 (TOM_{31c}), and BR-5 (TOM_{31c}) were confirmed to carry TOM_{31c}. EP-2 (TOM_{31c}) apparently carries a plasmid of similar size to TOM_{31c} (Table 11), but was shown by lack of hybridization to the tomABC probe to be unrelated (Table 9).

Table 11. TOM$_{31c}$ PLASMID BANDS FROM SELECTED TRANSCONJUGANTS VIA GEL ELECTROPHORESIS

Strain	Km^r	TOM$_{31c}$ band
HB101 (TOM$_{31c}$)	+	+
PR1$_{31}$ (TOM$_{31c}$)	+	+
249-2 (TOM$_{31c}$)	+	+
Presumed Transconjugants		
MFG-4 (TOM$_{31c}$)	+	+
MFE-2 (TOM$_{31c}$)	+	+
WS-23 (TOM$_{31c}$)	+	+
BR-5 (TOM$_{31c}$)	+	+
NFL-2 (TOM$_{31c}$)	+	+
NFG-2 (TOM$_{31c}$)	+	+
MFI-1 (TOM$_{31c}$)	+	+
UWL-1 (TOM$_{31c}$)	+	+
A2-1 (TOM$_{31c}$)	+	-
EP-2(TOM$_{31c}$)	+	+[1]

[1]Similar size to TOM$_{31c}$ but not related.

A summary of all attributes measured for the putative transconjugants is found in Table 12.

Table 12. EVALUATION OF PUTATIVE TRANSCONJUGANTS.

Putative Transconjugant	Selection[1]	Phe+ Km^r	Colonial morphology Similar to	Antibiotic profile similar to	TFMP	PCR2	Plasmid bands similar size as TOM_{31c}
MFG-4(T)	Km Amp	+	Recipient	Mixed	-	-	ND
NFG-2(T)	Km Amp	+	Recipient	Recipient	+	-	+
A2-1(T)	Km Amp	+	Recipient	Recipient	-	-	ND
MFI-1(T)	KmCm	+	Both	Recipient	+	+	+
EPI-1(T)	Phenol Pip	+	Recipient		-		ND
WFGATTB(T)	Phenol Pip	+	Recipient		-		ND
WFBL-4(T)	Phenol Pip	+	Recipient		-		ND
NFL-2(T)	Phenol Pip	+	Both		-		+

[1] Abbreviations: Km, Kanamycin sulfate; Amp, Ampicillin; Pip, Pipericillin; ND, None Detected
[2] PCR amplification of TOM_{31c} sequences

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4.   Survival of Transconjugants in Soil

The confirmed transconjugates containing TOM_{31c} were tested to evaluate their survival and ability to express Tom (i.e. degrade TCE) when exposed to stresses present in non-sterile environments.

The organisms WS-23 (TOM_{31c}), MF-4 (TOM_{31c}), and MFG-2 (TOM_{31c}) were grown overnight in 20 mL of R2A broth supplemented with 50ug/L Km, pelleted, and resuspended in 1x BSM. Six 250 mL Erlenmeyer flasks containing 100 grams of soil obtained at UWF. Forty mL of distilled water was prepared to serve as competitive environments for the transconjugates. Three of the flasks were amended with 1xBSM and a selective carbon source (20 mM glucose or ETOH). Two flasks were prepared for each strain. One received a selective carbon source, and the other did not. The inoculum was c.a. approximately 10^7cells/gram of soil (based on A_{600} extrapolation). Three uninoculated controls were run as well: two containing 20mM glucose and one with 20mM ETOH.

The nine flasks were then incubated at room temperature with rotary shaking at 200 rpm. After 12 days of incubation, 20mM glucose and 20mM ETOH were added to the appropriate flasks. Duplicate dilutions for plate counts were performed on the day of inoculation, and then twice a week for three and a half weeks. Cells were enumerated on R2A agar and 2mM phenol Km plates (Figure 29-31).

Cell counts on both phenol Km and R2A plates from slurries amended with a carbon source were slightly higher than cells obtained from unamended slurries for both inoculated and uninoculated flasks. It is impossible to determine what percentage of the cells enumerated were transconjugates without DNA hybridization.

Apparently none of the transconjugates rose to a level of dominance as indicated by these survival studies. Cell counts from inoculated flasks were similar to cell counts from uninoculated flasks when plated on both R2A and phenol Km agar with the exception of the uninoculated and unamended soil slurry. In this light, determination of bacterial population densities of total heterotrophs and kanamycin resistant phenol degraders is unrevealing for non-sterile soil studies.
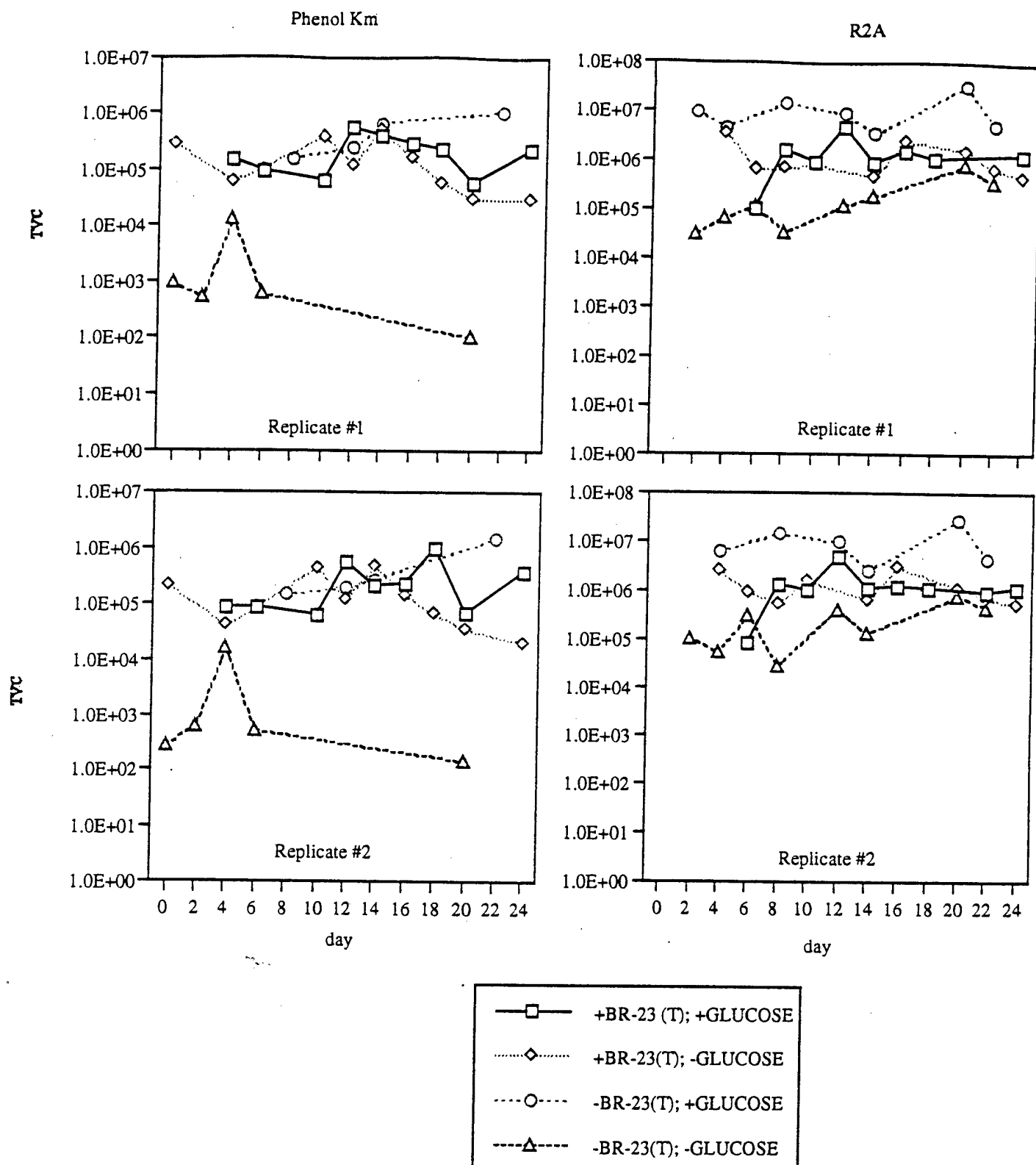
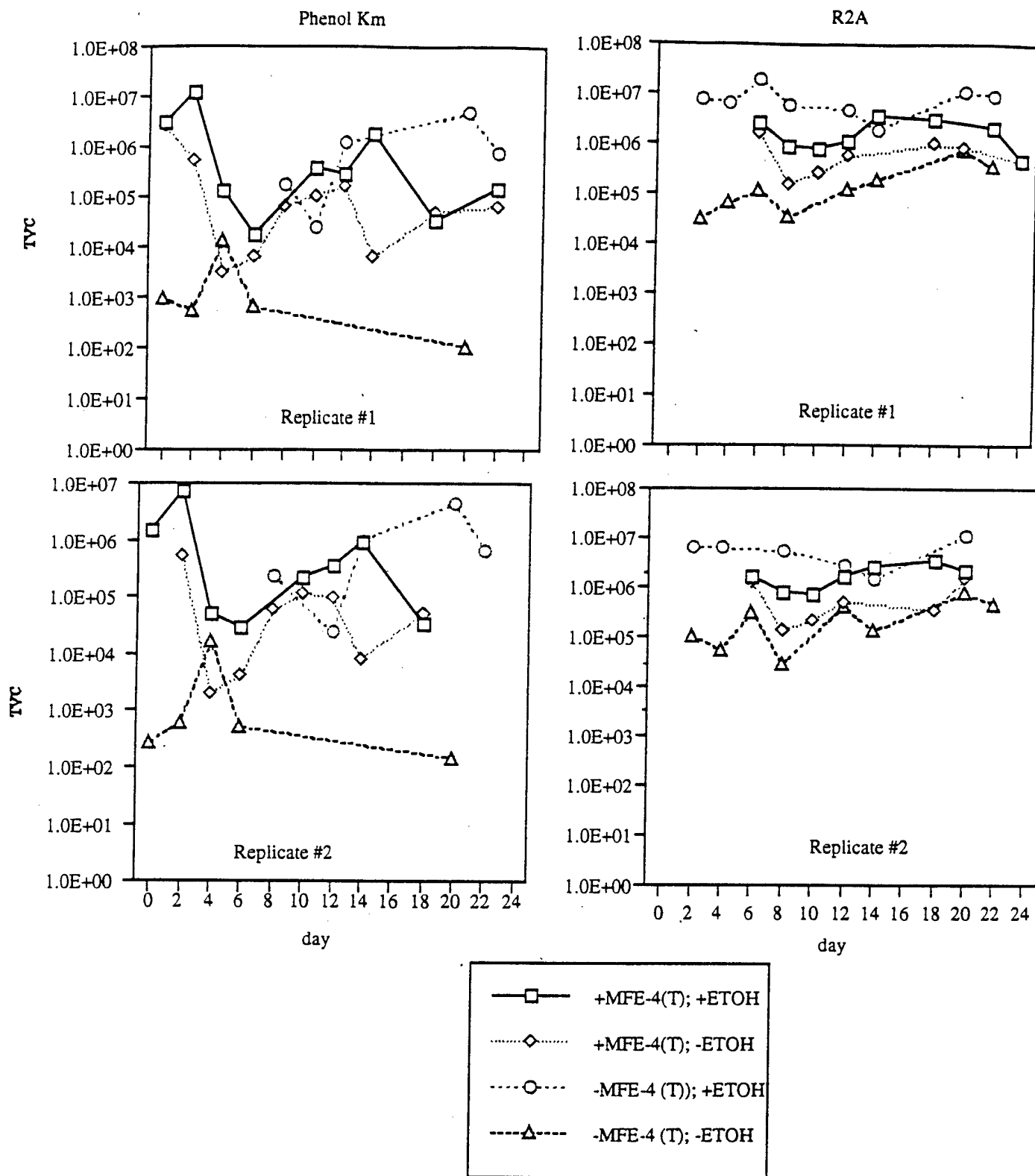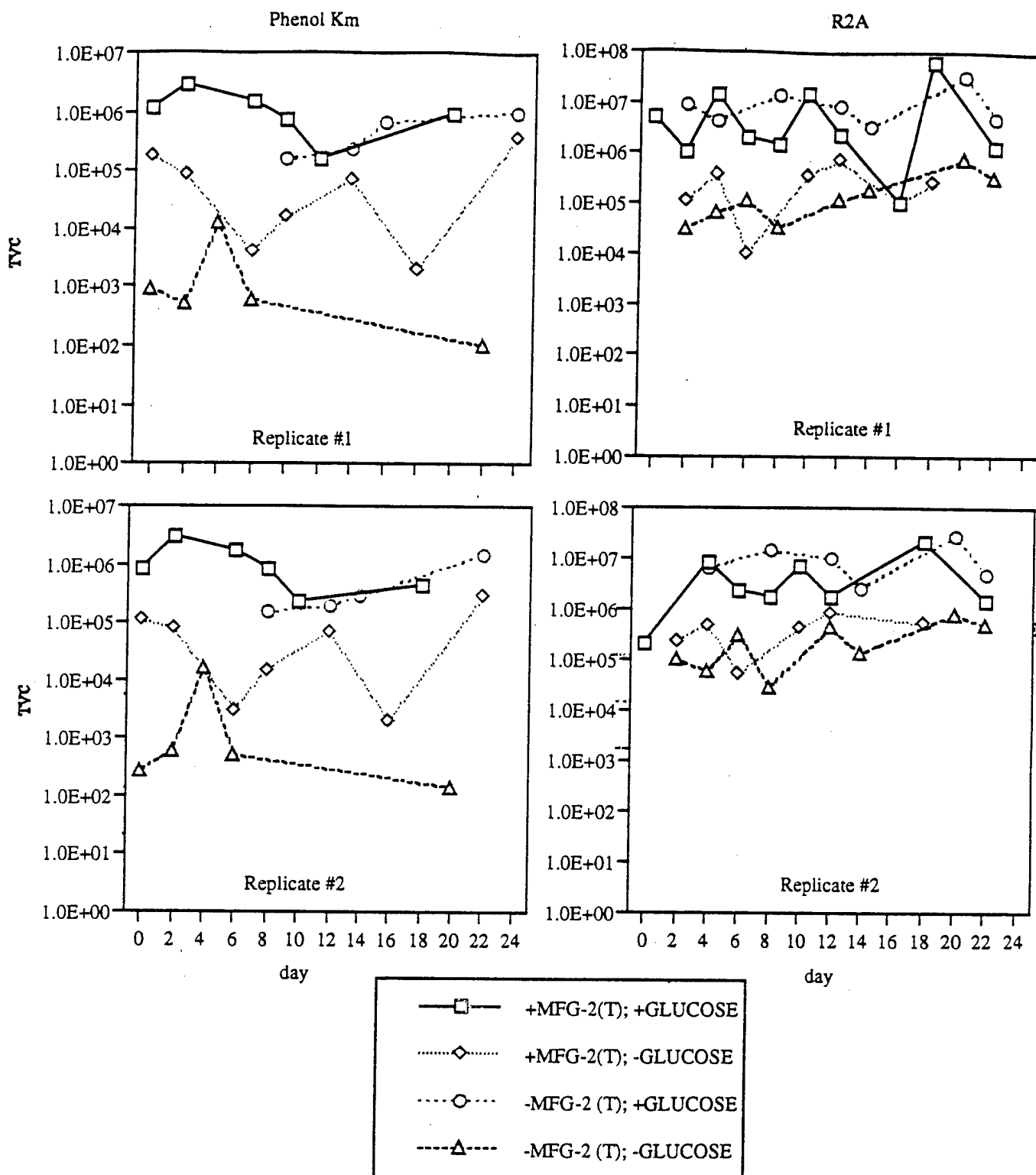Figure 28. Br-23 Survival in UWF Soil Slurries

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Figure 29.   MFE-4 (TOM$_{31c}$) Survival in UWF Soil Slurries

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Figure 30. MFG-2(TOM31c) Survival in UWF Soil Slurries

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5.   FAV Growth Curves at Various Temperatures

Growth curves were determined for the transconjugants to determine their growth potential in a relatively warm aquifer temperature of 15°C, compared to their growth at room temperature (22°C). The strains tested were MFG-2 (TOM$_{31c}$), NFG-2 (TOM$_{31c}$), BR-5 (TOM$_{31c}$), and WS-23 (TOM$_{31c}$).

MFG-2 (TOM$_{31c}$) and NFG-2 (TOM$_{31c}$) were grown overnight on 20 mM Glucose (60 mM C: 6 mM N: 30 mM P) and each added to two flasks containing 25 mL of the same medium to an A$_{600}$ between 0.05 - 0.09. BR-5 (TOM$_{31c}$), and WS-23 (TOM$_{31c}$) were grown overnight in R2A broth and added flasks containing 25 mL R2A broth to give a final A$_{600}$ between 0.05 - 0.09. The cultures were allowed to shake at either 22°C or 15°C and the A$_{600}$ monitored until stationary phases were achieved (Figure 32). A similar response was observed in all strains. Each yielded slower but substantial growth at 15°C. From these the curves corresponded to maximal doubling times were calculated for MFG-2 (TOM$_{31c}$), NFG-2 (TOM$_{31c}$), BR-5 (TOM$_{31c}$), and WS-23 (TOM$_{31c}$) as 2.5, 2.1, 1.1, and 1.2 hrs respectively at 22°C, and 10.7, 7.0, 2.5, and 12 hrs at 15 °C respectively.

Apparently all of the transconjugants growth was slowed significantly when forced to grow at 15°C, but BR-5 (the Borden aquifer isolate) was clearly capable of the most rapid growth at this temperature when fed R2A.

6.   Genetic Stability of TOM$_{31c}$ in Hosts: MF-4, MFG-2, BR-5, and WS-23

The stability of TOM$_{31c}$ in the four transconjugant host strains: MF-4 (TOM$_{31c}$), MFG-2 (TOM$_{31c}$), BR-5 (TOM$_{31c}$), and WS-23 (TOM$_{31c}$) was determined in the absence of km selection. Each strain was grown to stationary phase in R2A media and used to inoculate 25 mL flasks of R2A broth. After 50 generations, the final cell concentration was determined by plate counts on the isolates selective carbon substrate. The final percentage of Km^r colonies was determined by picking and transferring a representative number (>50) to R2A containing 50 μg/mL km. The final percentages of phenol-degrading colonies were determined by transferring the same colonies to BSM plates supplemented with 2 mM phenol. These determinations were performed in duplicate and are presented in Table 13.
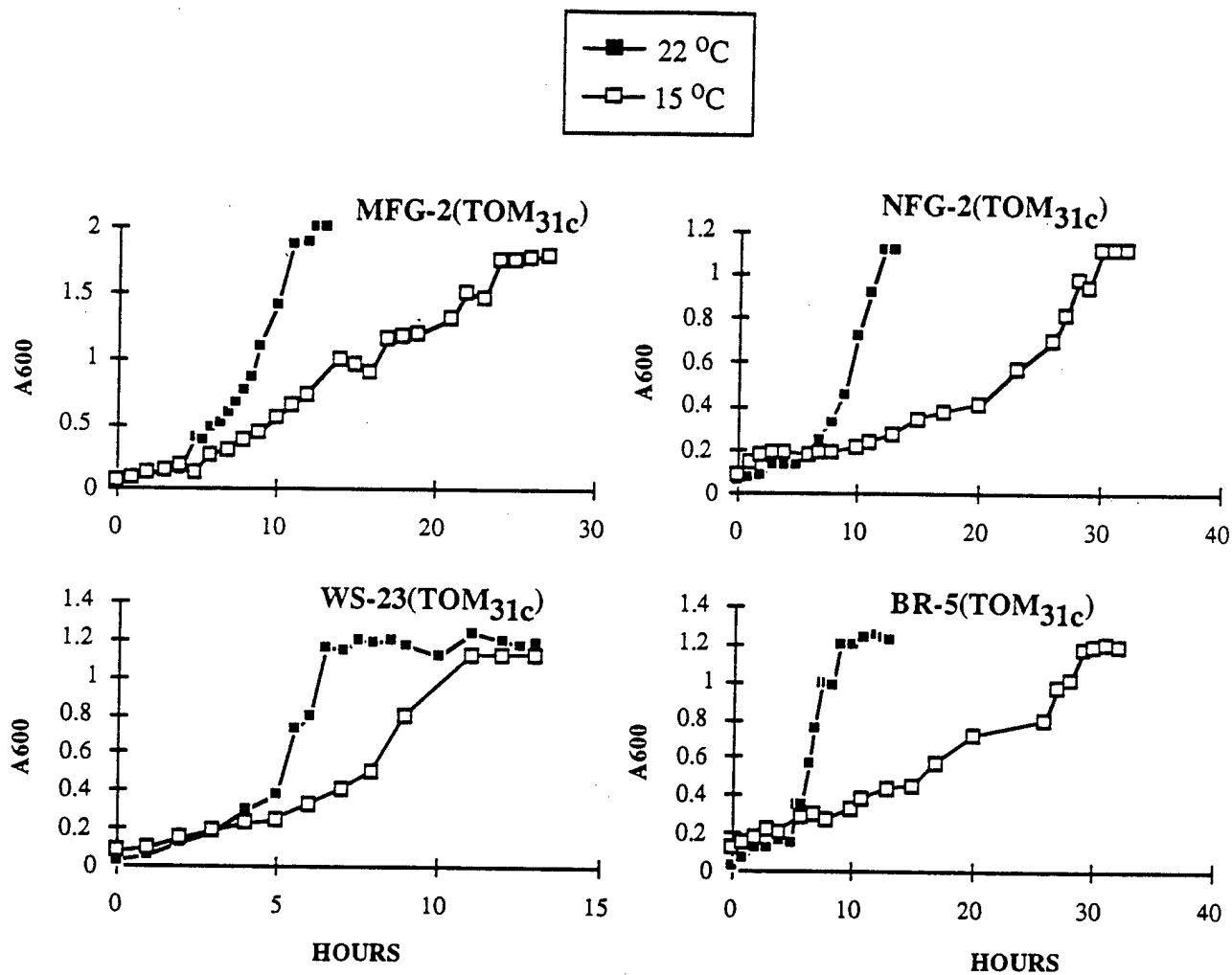
FIGURE 31. Transconjugant Growth Curves

The maintenance of the Km^r phenotype is a highly reliable marker for the presence of TOM$_{31c}$. Km^r BR-5(TOM$_{31c}$) failed to grow on phenol despite the presence of TOM$_{31c}$. The simplest interpretation for this observation is that the promoter for the neomycin resistance gene of Tn5 is recognized and expressed by BR-5, whereas the IS50 constitutive promoter is not. This is in keeping with the physical data and lack of TFMP oxidation and TCE degradation by this strain as well. The WS-23(TOM$_{31c}$) seems to exhibit a difference in that only ~70% of the kanamycin-resistant cells were capable of growing with phenol as the sole carbon source. I know of no reason why the Km^r and phenol degradation would not remain linked since neither were selected for. The Km^r would suggest stability of the plasmid, but failure of these isolates to grow on phenol suggests that the catabolic genes are no longer present or expressed. To further define this would require further investigation. Should WS-23(TOM$_{31c}$) prove to be of particular interest this should be pursued.

Table 13. PLASMID STABILITY IN FAV TRANSCONJUGANTS CONTAINING TOM$_{31c}$

Strain	cell concentration (10^8)	% of Km-res. cells	% of Cells that Degrade Phenol
MFG-2(TOM$_{31c}$)	17	100	100
	19	100	100
MF-4(TOM$_{31c}$)	Information not available		
BR-5(TOM$_{31c}$)	28	100	0
	32	100	0
WS-23(TOM$_{31c}$)	15	100	73
	16	100	69

SECTION IV

EFFECTIVENESS OF Tom CONSTITUTIVE FAV STRAINS IN NATIVE SOIL AND
GROUNDWATER

A.   INTRODUCTION

Having constructed these TCE-degrading transconjugant strains, the final phase of this
research project was to determine their ability to actually degrade TCE under environmental
conditions and to determine if the selective pressure for which the FAVs were selected would
aid in extending their activity and survival in such native soils. Transconjugants MFG-2
(TOM$_{31c}$), NFG-2 (TOM$_{31c}$), and MFI-1 (TOM$_{31c}$) were selected for these determinations because
of the superior degradative capabilities demonstrated under controlled conditions (Section III).

B.   MATERIALS AND METHODS

1.   Organisms and Culture Conditions

The bacteria and controls used in this section have already been described in the
preceding sections and include: MFG-2 (TOM$_{31c}$), NFG-2 (TOM$_{31c}$), MFI-1 (TOM$_{31c}$), and PR1$_{31}$
(TOM$_{31c}$). These bacteria were grown as indicated in the results.

2.   TCE Degradation Analyses

Air headspace analyses of suspended aquifer slurries (described in section III)
were used in this section.

3.   Molecular Techniques.

DNA:DNA hybridizations from colony pulls were performed as described in
Section III.

C.   RESULTS AND DISCUSSION

1.   Cell Concentration Effects on TCE Degradation in Aquifer Material

The ability of transconjugants to degrade TCE over an extended time period in unsterilized aquifer sediment without nutrient amendments was determined at a variety of cell concentration. The object was to determine the "effective dose" of bacteria necessary to elicit a detectable degradative response in these untreated aquifer materials.

The transconjugants MFG-2 (TOM_{31c}) and NFG-2 (TOM_{31c}) were grown overnight in 20 mM glucose, 1xBSM, and MFI-1 (TOM_{31c}) was grown overnight in R2A broth. The overnight cultures were pelleted and resuspended in UWF aquifer water. Ten 165 mL serum bottles containing 20 grams (dry weight) of sediment obtained from an uncontaminated aquifer in Pensacola were supplemented with 5 mL of liquid consisting of resuspended cells, carbon, nitrogen, and phosphorus. The serum bottles were sealed with Teflon® septa, crimped shut, and TCE was added via syringe. The exact concentrations of cells and chemical amendments are given in Table 14.

Table 14.   CELL CONCENTRATION EFFECTS IN SEDIMENT AMENDMENTS

Strain	cells/mL	Amendment (C:N:P)	TCE[1]
MFG-2 (TOM_{31c})	2.0×10^9	4 mM glucose; 12: 1.2: 6	≈ 1000 μg/L
	1.7×10^8	4 mM glucose; 12: 1.2: 6	≈ 1000 μg/L
	6.0×10^7	4 mM glucose; 12: 1.2: 6	≈ 1000 μg/L
NFG-2 (TOM_{31c})	2.0×10^9	4 mM glucose; 12: 1.2: 6	≈ 1000 μg/L
	5.6×10^7	4 mM glucose; 12: 1.2: 6	≈ 1000 μg/L
	3.8×10^7	4 mM glucose; 12: 1.2: 6	≈ 1000 μg/L
MFI-1 (TOM_{31c})	2.0×10^7	0.02% IGEPAL; 11: 1.1: 5.5	≈ 1000 μg/L
	1.4×10^6	0.02% IGEPAL; 11: 1.1: 5.5	≈ 1000 μg/L
No cells control #1	none	4 mM glucose; 12: 1.2: 6	≈ 1000 μg/L
No cells control #2	none	0.02% IGEPAL; 11: 1.1: 5.5	≈ 1000 μg/L

[1]The concentration of TCE calculated as the concentration in the vapor phase.

The serum bottles were allowed to shake at 15°C for approximately two weeks in which 500 μL air headspace samples were taken one-two times per day and analyzed via direct on column injection to the GC. Near the end of the assay, carbon, nitrogen, phosphorus and oxygen were added to the serum bottles to determine if such supplementation would result in further degradation of the TCE.

NFG-2 (TOM$_{31c}$) added to native aquifer material at 2.0 x 10^9 cells/mL successfully degraded all detectable TCE within 24 hours and continued to degrade following a second TCE addition almost 2 weeks later. NFG-2 (TOM$_{31c}$) at 5.6e7 cells/mL and 3.8e7 cells/mL resulted in TCE degradation over a 10-day period. Degradation after 10 days was not detected.

MFG-2 (TOM$_{31c}$) added to native aquifer material at 2.0e9 cells/mL also appeared to degrade all detectable TCE in 24 hours and again following a second TCE addition almost 2 weeks later. MFG-2 (TOM$_{31c}$) at 1.7e8 cells/mL and 6.0e7 cells/mL did not degrade TCE during the same 2-week incubation. Because of the apparently large discrepancy between the results of the two higher cell concentrations (and because of the survey nature of this experiment allowing only a single test system per cell amendment) the disappearance of TCE at the highest cell concentration must be viewed skeptically and could in fact be due to a leaky bottle.

MFI-1 (TOM$_{31c}$) added to native aquifer material at 2.0e7 cells/mL and 1.4e6 cells/mL did not degrade TCE during this two week incubation. In later experiments however, MFI-1 proved particularly effective at TCE degradation in native soils.

2.   TCE Degradation in Aquifer Material with Constant Addition of Nutrients.

The ability of these FAVs to survive and degrade TCE in native aquifer material constantly amended with carbon, nitrogen, phosphorous, and oxygen additions was determined. In this experiment the headspace measurements were taken from triplicate samples to avoid the possibility of erroneous degradation measurements.

The transconjugants MFG-2 (TOM$_{31c}$) and NFG-2 (TOM$_{31c}$) were grown overnight in 20 mM glucose and 1xBSM, MFI-1 (TOM$_{31c}$) was grown overnight in R2A broth, and PR1$_{31}$ (TOM$_{31c}$)was grown overnight in 20 mM lactate and 1xBSM. The overnight cultures were pelleted and resuspended in UWF aquifer water. Twenty-four 165 mL serum bottles (performed in triplicate) containing 20 grams (dry weight) of sediment obtained from an

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uncontaminated aquifer in Pensacola and 5 mL of water containing resuspended cells, carbon, nitrogen, and phosphorus, Table 15.

Table 15. CELL AND NUTRIENT SEDIMENT AMENDMENTS

Strain	cells/mL	Amendment (selection; C:N:P)	TCE
MFG-2 (TOM$_{31c}$)	2.0×10^8	1mM glucose; 6: 0.6: 3	≈ 1000 μg/L
NFG-2 (TOM$_{31c}$)	2.0×10^8	1mM glucose; 6: 0.6: 3	≈ 1000 μg/L
MFI-1 (TOM$_{31c}$)	2.0×10^8	0.002% IGEPAL; 1.2: 0.1: 0.6	≈ 1000 μg/L
PR1$_{31}$ (TOM$_{31c}$)	3.2×10^7	1mM lactate; 3: 0.3: 1.5	≈ 1000 μg/L
No cells added	none	1mM glucose; 6: 0.6: 3	≈ 1000 μg/L
No cells added	none	0.002% IGEPAL; 1.2: 0.1: 0.6	≈ 1000 μg/L
No cells added	none	1mM lactate; 3: 0.3: 1.5	≈ 1000 μg/L

The serum bottles were shaken at 15°C for the duration of the experiment (approximately 2 weeks) during which time 500 μL air headspace samples were taken 1-2 times daily for GC analysis. The C:N:P amendments were made (glucose: 25 μL 1 M stock; N,P: 83 μL stock at 180 mM nitrogen 900 mM phosphate (40 mM (NH$_4$)$_2$HPO$_4$, 40 mM NH$_4$NO$_3$, 20 mM NH$_4$Cl, 860 mM K$_2$HPO$_4$) at the same ratios listed above as well as 2 mL of oxygen were added every other day to the serum bottles.

MFG-2 (TOM$_{31c}$) (Figure 28) did not degrade TCE in native aquifer material at 2.0×10^8 cells/mL. In replicate 1 a small amount of TCE disappears; however, no degradation was seen in either replicate 2 or 3. After 35 days of incubation the TCE concentration was measured again and no significant change in the concentration was observed in any of the replicate microcosms.

The FAV MFI-1 (TOM$_{31c}$) (Figure 29) at 2.0×10^8 cells/mL was able to degrade ≈ 1000 μg/L of TCE to 0 ug/L after nine days of incubation; however, upon the second addition of TCE the cells were unable to continue the degradation. After 35 days of incubation, no more TCE was degraded.

The FAV NFG-2 (TOM$_{31c}$) (Figure 30) at 2.0×10^8 degrade ≈ 1000 μg/L of TCE to 0 ug/L after only 3 days of incubation and continued to degrade after a second addition of TCE. The third replicate degraded the second addition of TCE to zero by the eighth day and
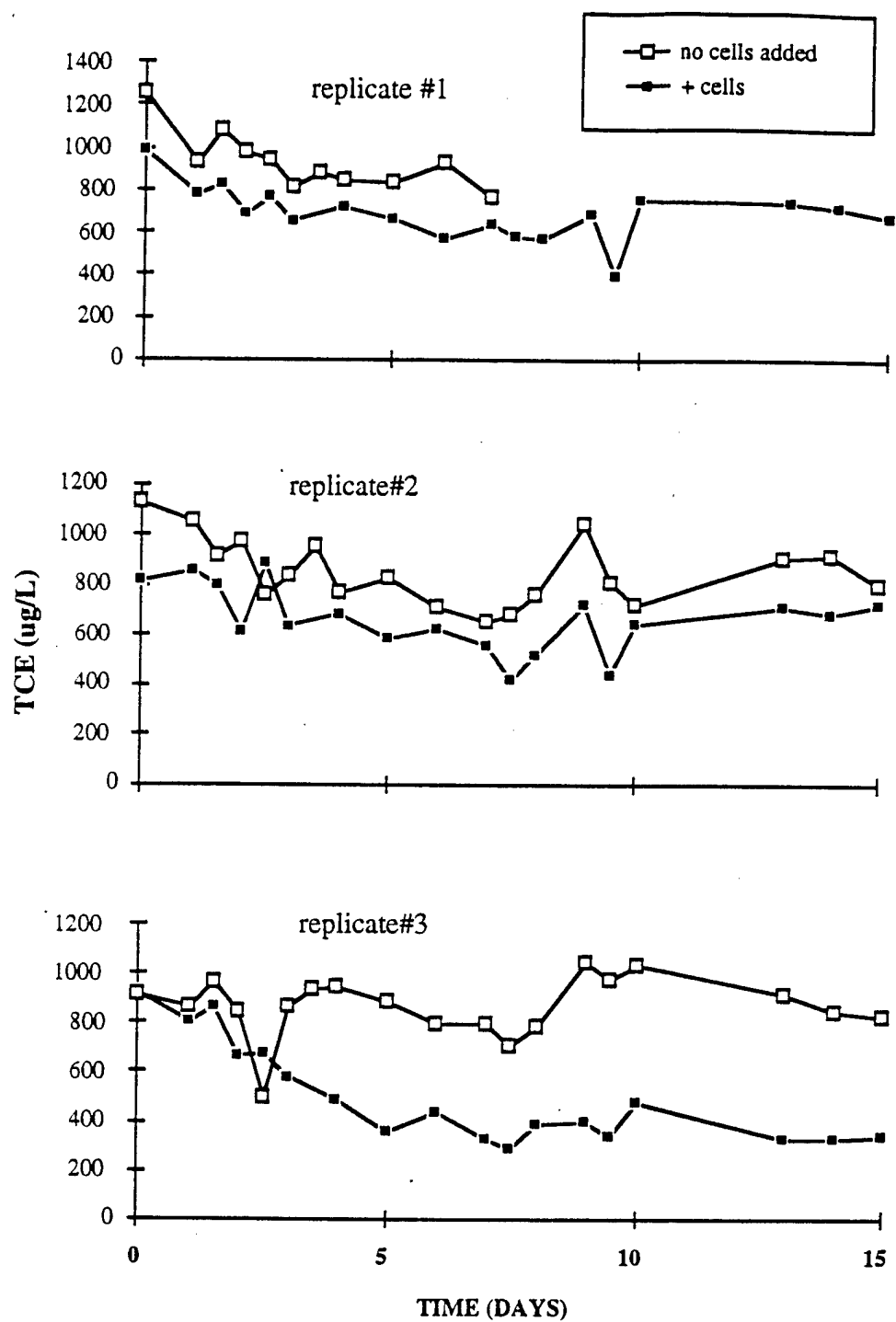
82

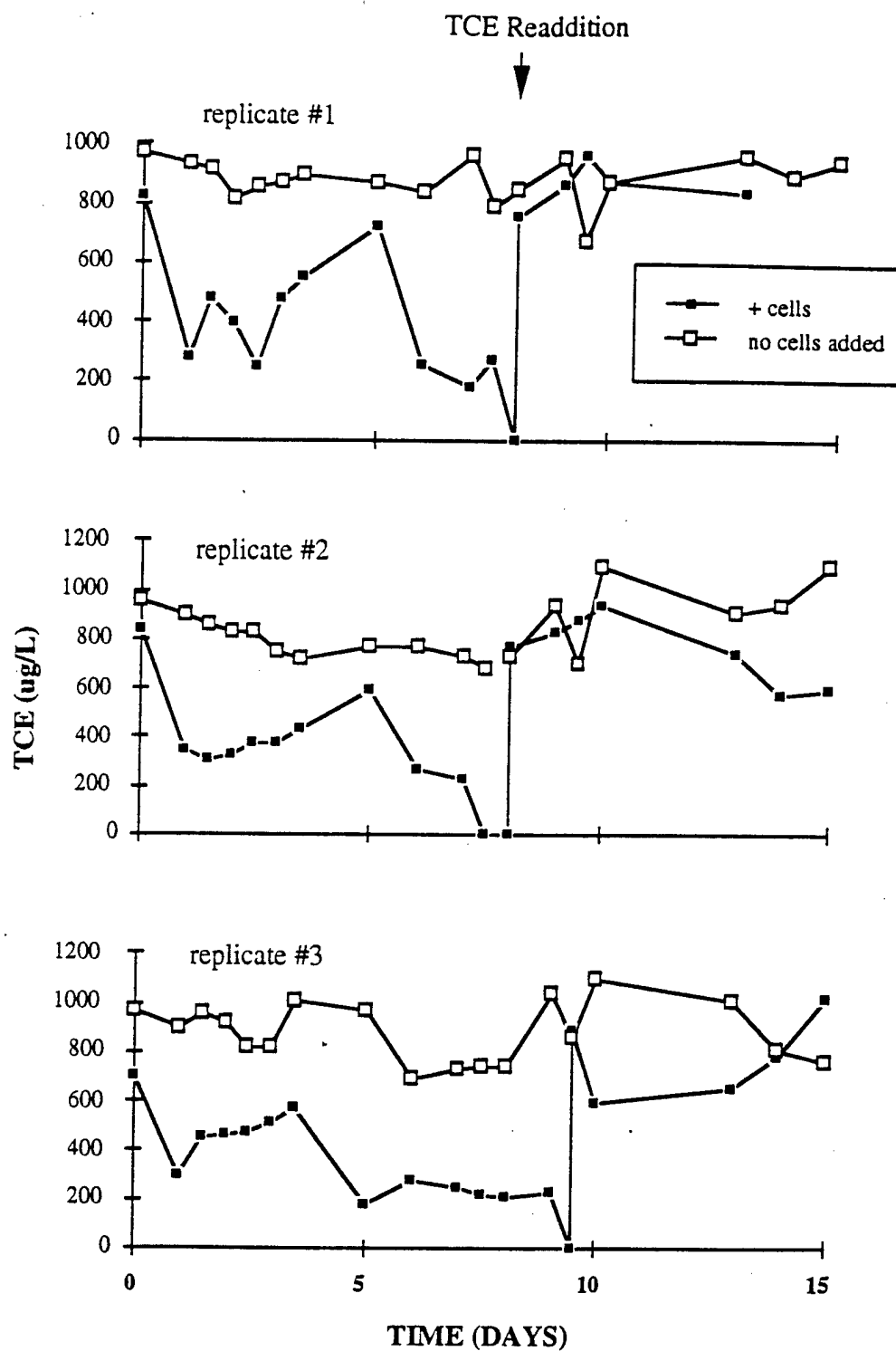**Figure 32.** TCE Degradation by MFG-2(TOM$_{31c}$) in Aquifer Materialwith Constant Nutrient Addition.

Figure 33. TCE Degradation by MFI-1 (TOM$_{31c}$) in Aquifer Material with Constant Nutrient Addition
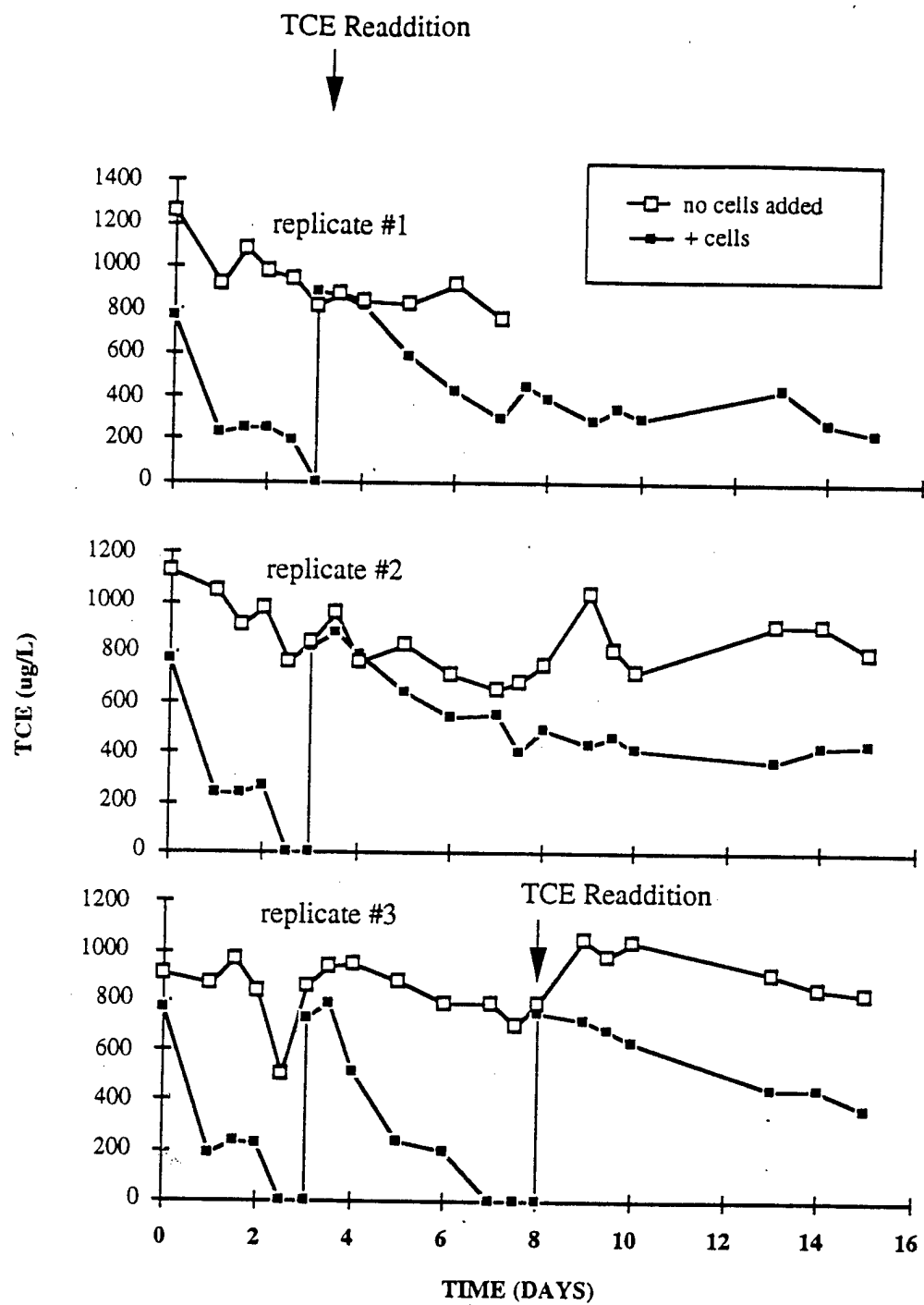
Figure 34. TCE Degradation by NFG-2(TOM$_{31c}$) in Aquifer Sediment with Glucose Addition.

continued to degrade after a third TCE addition. Replicates 1 and 2 degraded the second TCE addition to zero sometime after the 15th day because on the 35th day of incubation, the TCE concentration was measured at zero.

PR1$_{31}$ (TOM$_{31c}$) (Figure 36) showed little demonstrable ability to degrade TCE in unsterilized aquifer material at 3.2×10^7 cells/mL.

3.   Transconjugant Survival In Native Sediments

Sediment test systems were inoculated once only with a known concentration of selected TOM$_{31c}$ transconjugants and pulsed at regular intervals with predetermined amounts of C, N, P, and O$_2$. The sediment systems were constructed as described above (section C 2). The added liquid amendment included the initial cell load (about 2×10^8 cells/mL), a C:N:P pulse at the ratio of 10:1:5, TCE c.a. at a final liquid concentration (as if all were in liquid phase) of 10 μM, and trace metals. The water was untreated UWF aquifer H$_2$O. The carbon sources used were glucose (1mM), IGEPAL (0.002%), and Lactate (1mM). Each sediment system was pulsed with the appropriate amount of nutrients each day that a sample was taken. This worked out to a pulsing/sampling schedule of Days 0, 2, 4, 6 , 8, 10, 13, 15, and 19 (systems were pulsed late the night of Day 19, samples were taken early the morning of Day 20). A final pulse was delivered on Day 27 although no sample was taken at that time. In addition, an appropriate volume of headspace was replaced with pure O$_2$ gas to simulate the volume removed for TCE analyses described in section C 2, and to ensure that enough O$_2$ was available for oxidation of the added carbon.

a.   Selective Plate Enumeration

The transconjugants to be tested in these systems were NFG-2 (TOM$_{31c}$), MFG-2 (TOM$_{31c}$), and MFI-1 (TOM$_{31c}$). The systems with the two glucose isolates (NFG-2 and MFG-2) were pulsed with 1 mM glucose while the one with the IGEPAL isolate was pulsed with 0.002% IGEPAL. A parallel system inoculated with PR1$_{31}$(TOM$_{31c}$) (to approximately 3.2×10^7 cells/mL) served as a positive control, and was pulsed with 1 mM lactate. Uninoculated sediment systems represented baseline populations. The population of heterotrophic microorganisms, as well as those which were both phenol-utilizing and Km^r were monitored using R2A and selective agar (Phe/Km) plates. In addition, the population of Tn5

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containing cells were quantified by colony DNA blotting those Phe/Km plates which were countable with a radiolabled Tn5 probe.

Total heterotrophs and Km^r heterotrophs were enumerated from these test sediments with chemical amendments but without added transconjugants These control results are depicted in Figures 37-39. In these Figures the population measurements were performed on sediments pulsed with 1 mM glucose, 0.002% IGEPAL and 1 mM lactate respectively, but no bacteria. Figures 35, 36, 37 and 38 each received similar chemical amendments in addition to the bacteria: $PR1_{31}$ (TOM_{31c}), NFG-2 (TOM_{31c}), MFG-2 (TOM_{31c}), or MFI-1 (TOM_{31c}) respectively.

Preexisting phenol degrading Km^r bacterial populations were detected at about 1×10^5 cfu/mL water. These populations increased steadily to about 1×10^8 by the end of the 20 day experiment, which effectively represented the total heterotrophic count enumerated on R2A. Interestingly, the R2A heterotrophic population did not seem to respond to these substrate additions. This probably just reflects the upper carrying capacity of these sediments. The cause of this upper boundary remains unknown, but probably includes bacterial grazers which simply crop everything above this concentration.

The second observation is that the added bacteria were immediately detectable at approximately 1×10^8 cfu/mL on day 0 for $PR1_{31}$ (TOM_{31c}), and NFG-2 (TOM_{31c}) and MFI-1 (TOM_{31c}). MFG-2 (TOM_{31c}) was never recoverable in numbers above the background phenol/kanamycin counts. This may reflect a lower inoculum than intended, or just a poorer recovery of these bacteria on phenol/kanamycin medium.

b.   DNA Colony Hybridization

Also shown in Figures 37-43 are the results of colony hybridization studies with these sediments during the first 20 days of operation. The Day 0 samples were taken before inoculation with the respective TOM_{31c} carrying strains. Several conclusions may be drawn from this data:

The fact that the uninoculated controls receiving glucose, IGEPAL or lactate produced Km^r phenol degrading populations over the same time course and to approximately the same level as the transconjugant and PR131 inoculated sediments. However, unlike these inoculated sediments they never produced a population that hybridized to the Tn5 probe.
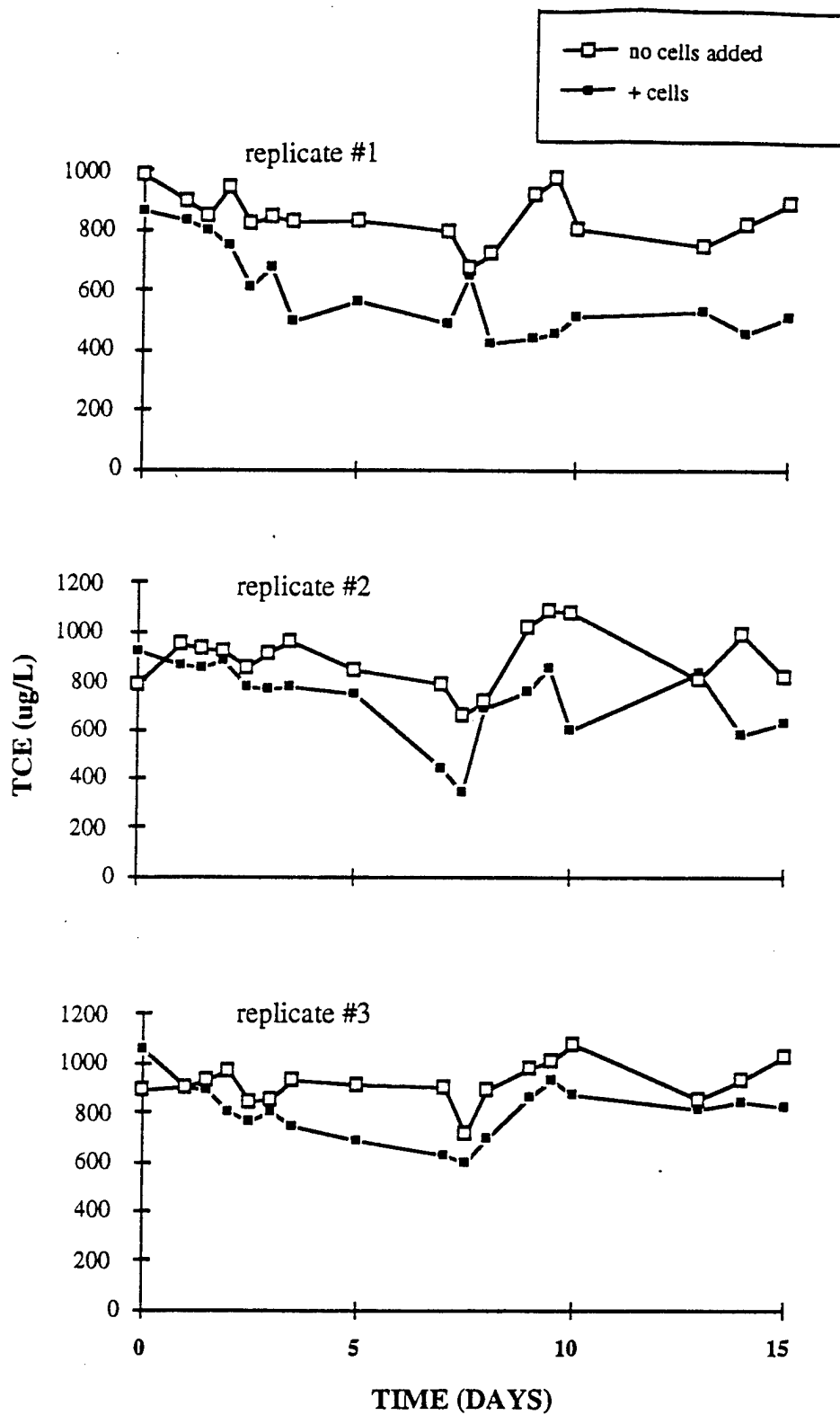
Figure 35. TCE Degradation by PR1$_{31}$ (TOM$_{31c}$) in Aquifer Sediment with Constant Nutrient Addition

Not all of the strains survived to the same extent. $PR1_{31}$ (TOM_{31c}), NFG-2 (TOM_{31c}) and (in a somewhat slower response) MFI-1 (TOM_{31c}) all produced populations that appeared stable through the last half of the 20 day monitoring period with respect to the Tn5 hybridizing population. These populations (unlike the uninoculated controls and the MFG-2 inoculated test) seemed to make up a consistent 1-10% of the total Km^r phenol degrading populations measured during the latter half of the experiment.

MFG-2 (TOM_{31c}) did not survive well at all according to this sediments inability to produce any detectable Tn5 hybridizing colonies.

$PR1_{31}$ (TOM_{31c}), NFG-2 (TOM_{31c}) and MFI-1 (TOM_{31c}) Tn5 hybridizing colonies seemed to increase over the treatment period in what would presumably be a response to the FAV selective carbon sources applied.
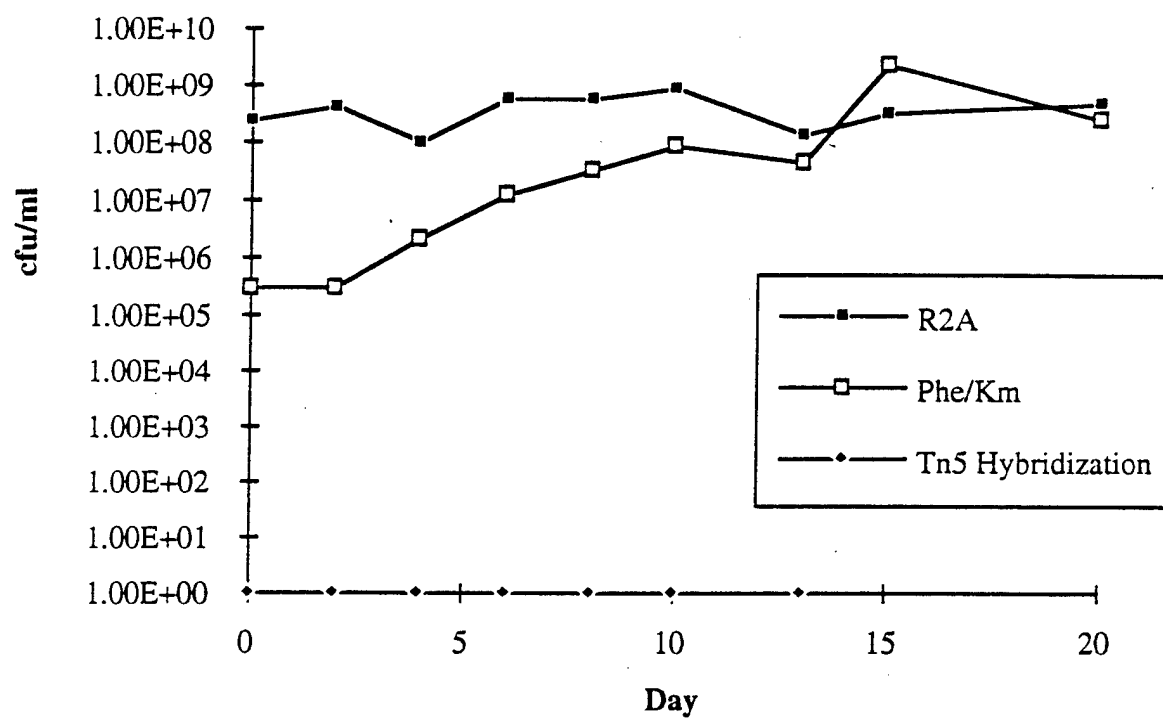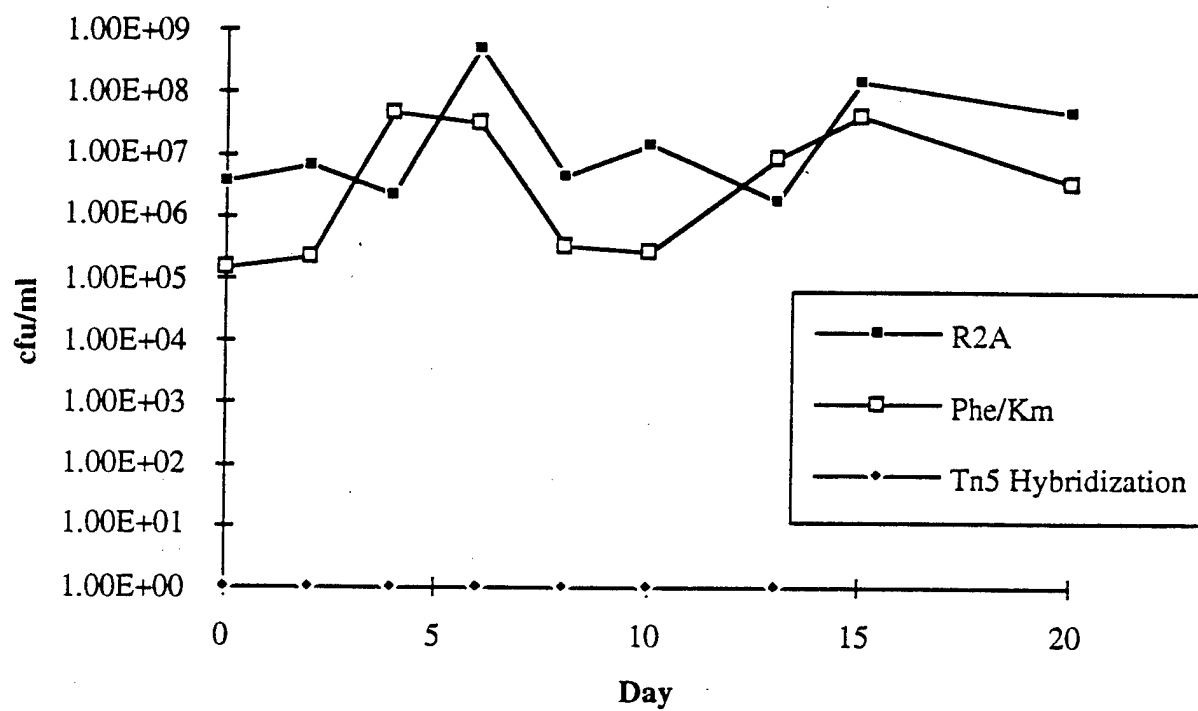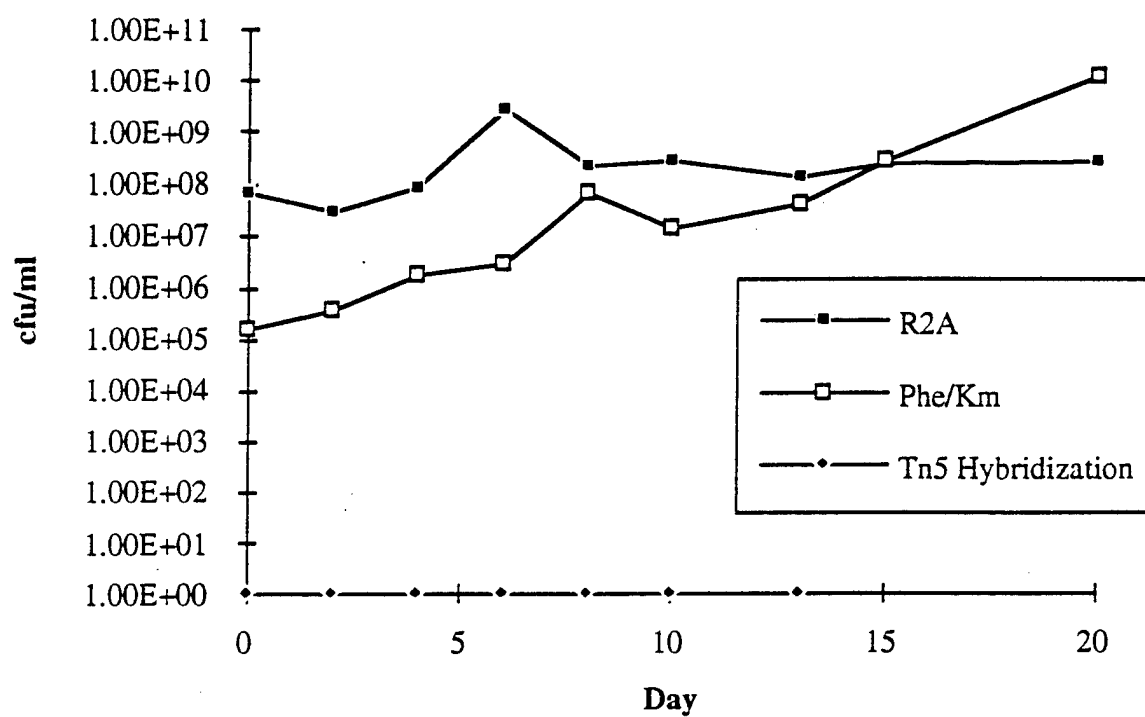
Figure 36. Bacterial populations in sediment pulsed with Glucose
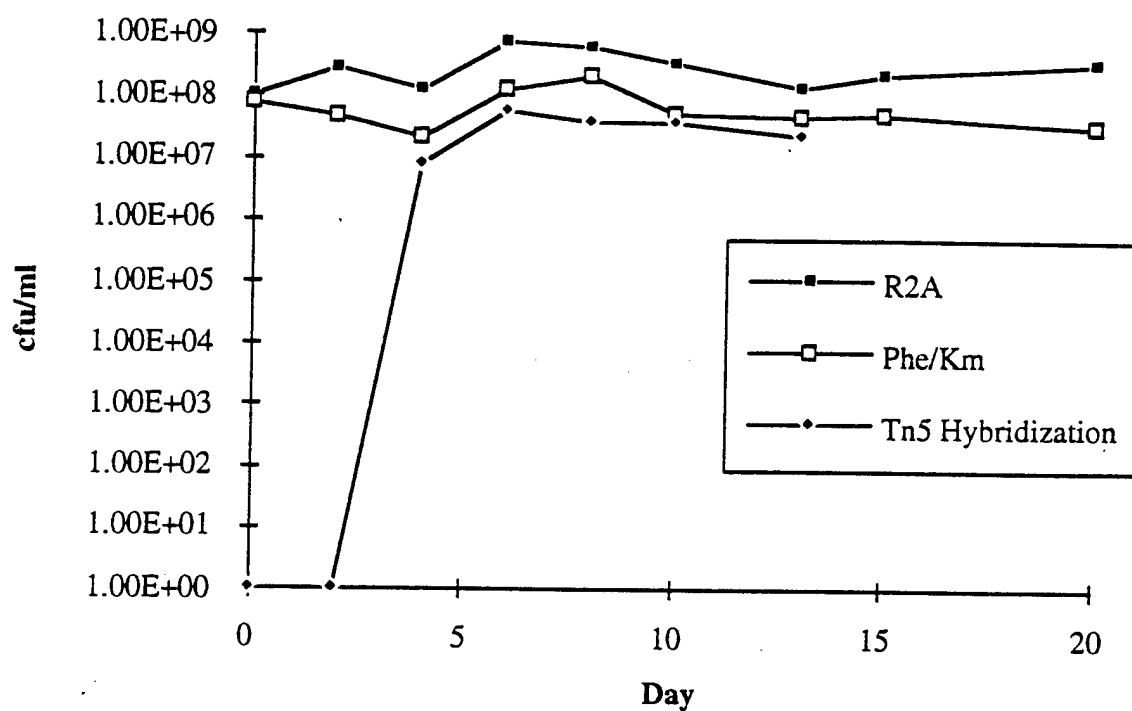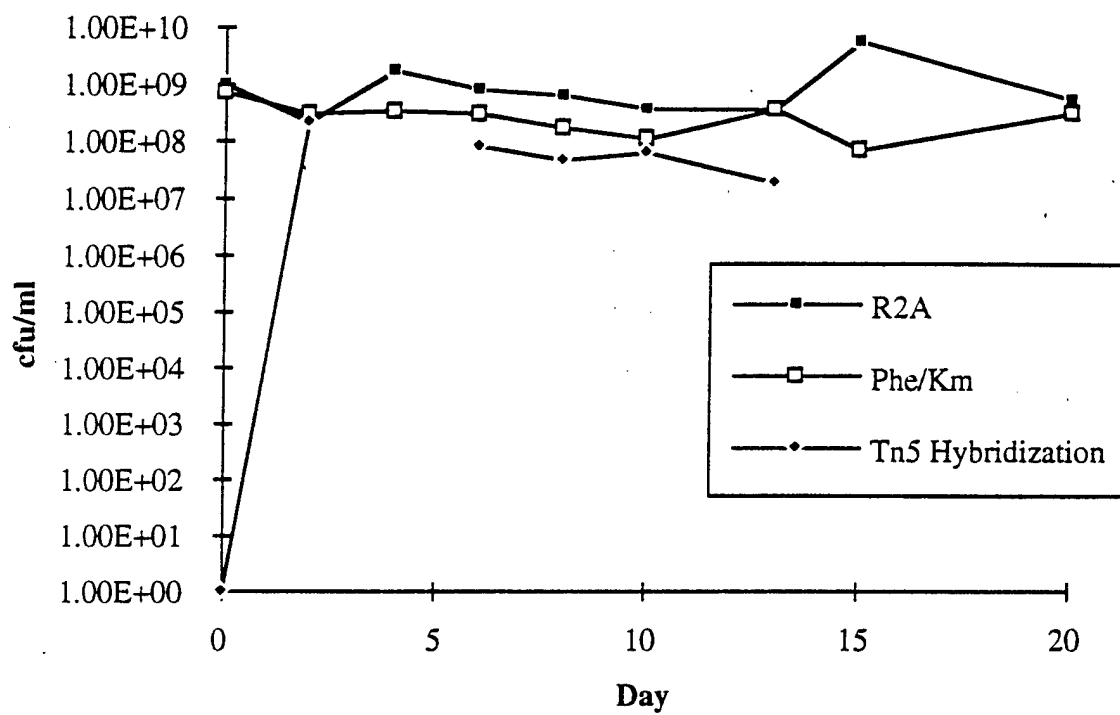
**Figure 37. Bacterial Populations in Sediment Pulsed with 0.002%
IGEPAL**

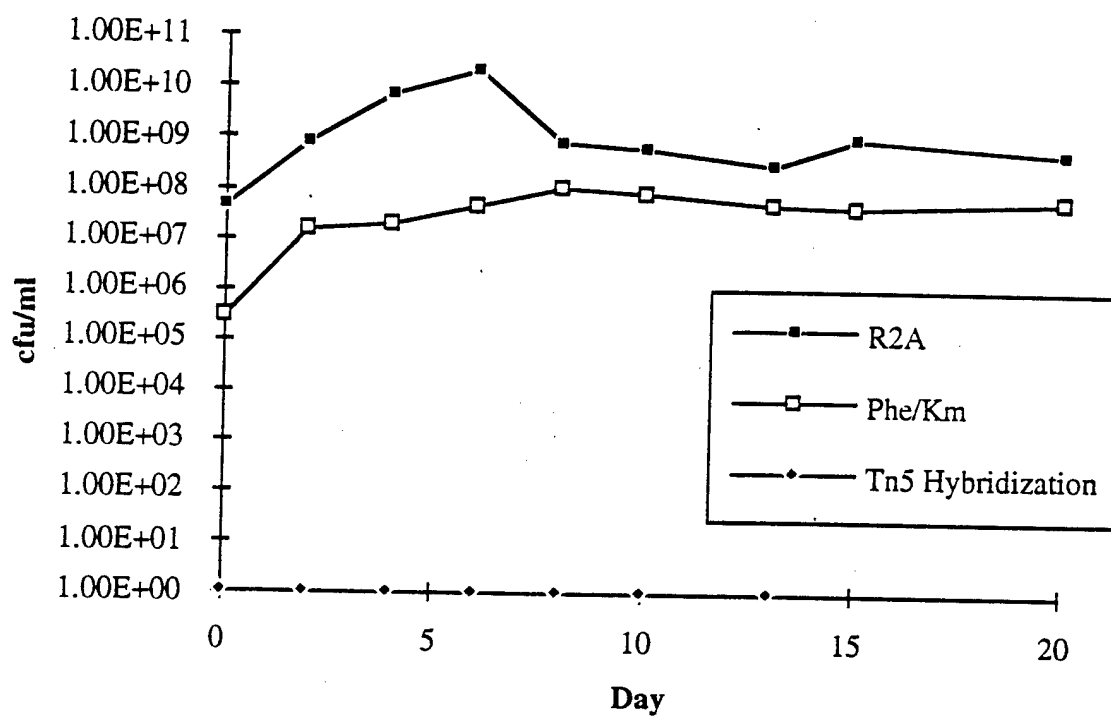**Figure 38. Bacterial Populations in Sediment Pulsed with 1mM Lactate**
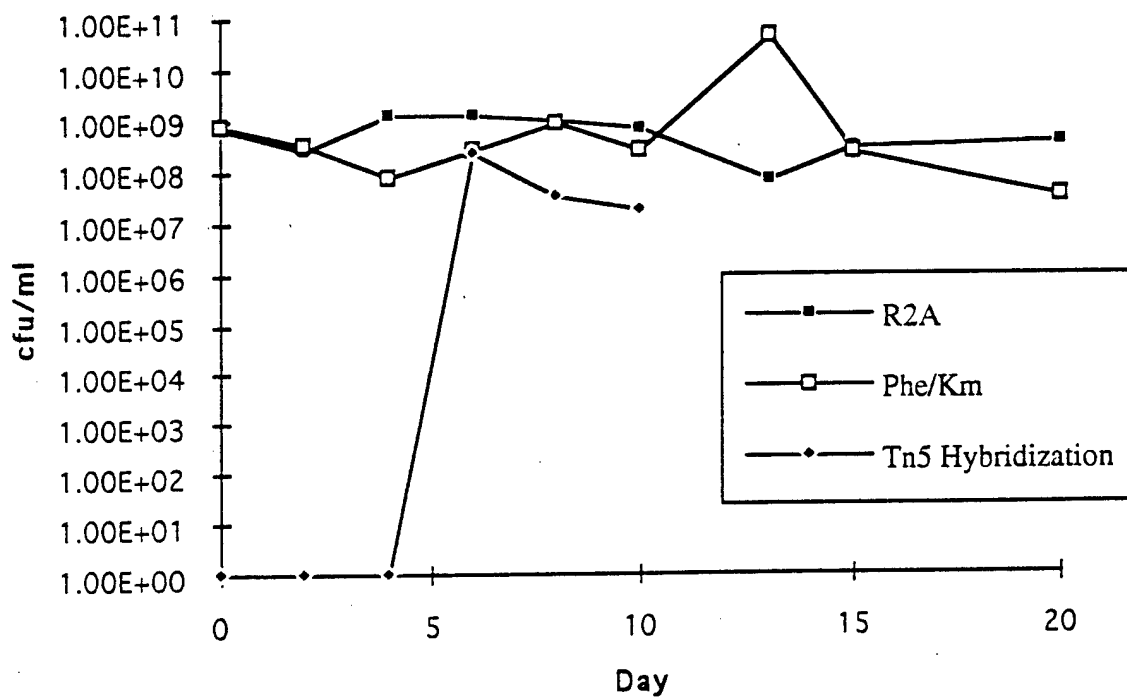
Figure 39. Bacterial Populations in Sediments Amended with PR1$_{31}$ (TOM$_{31C}$) and Pulsed with 1mM Lactate

Figure 40. Bacterial Populations in Sediment Amended with NFG-2 (TOM$_{31c}$) and Pulsed with 1mM Glucose.

Figure 41. Bacterial Populations in Sediments Amended with MFG-2 (TOM$_{31C}$) and Pulsed with 1mM Glucose.

Figure 42. Bacterial Populations in Sediments Amended with
MFI-1 (TOM$_{31}$C) and Pulsed with 0.002% IGEPAL.

SECTION V.

CONCLUSIONS

A fundamental problem with the utilization of $PR1_{31}$ (or any of the other constitutive
TCE degrading strains generated from *Burkholderia cepacia* G4) is that they have failed to
demonstrate appreciable activity in native aquifer matrices or bioreactors. The major cause of
this limitation is our inability to directly select this cometabolizing strain from the background of
native organisms competing for the source of carbon and energy needed for the cometabolic
process. Since there was no conscious design or selection of the original G4 isolate (or the
subsequent Tom constitutive derivatives) for survival and activity in TCE-contaminated
environments, its capacity to perform under such conditions was highly problematic.
Unfortunately, in several trials we have recently determined that these strains are not very
selectable under the most selective conditions we could design (in both soil columns and
bioreactors).

Our goal was therefore, to create other constitutive TCE degraders that could act as
FAVs and thus be selected under environmental conditions to predominate and degrade TCE *in
situ*. The results of this study indicate that specific bacterial strains can retain TOM_{31c},
constitutively express Tom and positively respond to selection pressure.

TOM_{31c} was successfully transferred to several aquifer isolates. In most instances, the
constitutive expression of Tom was maintained by these new bacterial hosts. Few of these
bacteria were more stable or better able to express the degradative phenotype under non-sterile
conditions (sealed sediment bottles at 15 °C). However two isolates demonstrated an equal or
greater capacity to survive in these native sediments and an enhanced capacity to degrade TCE:
NFG-2 (TOM_{31c}) and MFI-1 (TOM_{31c}). NFG-2 (TOM_{31c}) responded well to glucose addition
and both survived and demonstrated TCE degradative capacity for the longest of any in this test:
10 days. MFI-1 (TOM_{31c}) was the second best with demonstrated survival and continued TCE
degradation in the non-sterile systems for 5-7 days. Both strains clearly outperformed the parent
strain $PR1_{31}$ which acted as the donor of TOM_{31c} in terms of activity, though all three strains
demonstrated similar survival characteristics. Transconjugant MFG-2 (TOM_{31c}) performed very
poorly with neither survival nor activity demonstrated in these non-sterile sediments. Our
IGEPAL-degrading strain did not undergo as positive a selection as reported for another strain
in the literature (5). This is probably because our strain does not respond as well (i.e. does not

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grow as fast or tolerate as high a concentration) to IGEPAL. Despite this, the use of both IGEPAL and glucose seem warranted since both MFI-1 (TOM$_{31c}$) and NFG-2 (TOM$_{31c}$) both clearly outperformed the parent strain: PR1$_{31}$.

SECTION VI

RECOMMENDATIONS

We have demonstrated the ability to generate native bacterial isolates that can survive and undergo selection in natural matrices while maintaining the constitutive expression of Tom. It is now feasible to construct such FAVs either tailored to a specific site and selective nutrient combination or to generate a pool of such organisms that can be used to obtain the "best" candidate for a given site.

Other more selectable candidates should be sought and their suitability as stable hosts for TOM_{31c} determined. Also, the selectable hosts identified so far (NFG-2 and MFI-1) should next be transformed with the non-$Tn5$ containing constitutive TCE-degrading plasmid TOM_{301c}, and tested further for the most ideal selective conditions.

This study has merely explored the most obvious conditions and modes of delivery. A more realistic aquifer situation should be used to evaluate the true potential of these transconjugants. Once determined the TOM_{301c} bearing derivatives could be tested. Because of the absolutely nonrecombinant nature of TOM_{301c}, these isolates would be available for immediate release in an *in situ* field trial.

One of the more puzzling features of $PR1_{31c}$ is the apparent contradiction between its apparently good survivability and its relatively poor activity *in situ*. The two transconjugants that survived well (as determined by $Tn5$ hybridizable colonies) consequently were capable of extended TCE degradation as well. The poor survivor also (not surprisingly) did not degrade TCE. These results point out the need to better understand the physiology of the bacterium expected to perform the cometabolic task under the conditions it will have to perform it. Clearly survival is not an adequate predictor of performance.

Given the alternatives for remediation of TCE-contaminated aquifers such a survivable, selectable, constitutive degrader should prove of great value, and information gathered on the physiological state of such bacteria *in situ* would be of use in any bioaugmentation scenario.

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